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(54) Title: NUCLEIC ACIDS FOR TREATING OBESITY

(57) Abstract

A nucleic acid molecule is provided that can be expressed in a host cell to produce a biologically active ob polypeptide that can effectively inhibit food intake and/or weight gain. Vectors and host cells containing the nucleic acid molecule are also provided, as well as methods for producing the ob protein and other ob polypeptides, methods of induction of the production of the ob polypeptides, such as by *in vivo* or *ex vivo* gene therapy, and methods for inhibition of food intake and/or weight gain. Further provided are antibodies to the ob polypeptides and methods of using such antibodies, such as for identification or detection of other ob polypeptides or homologs thereof, and for inhibition of ob polypeptide activity. A method for identification, detection, or isolation of an ob receptor is provided as well as methods for production of antibodies to the ob receptor. The antibodies and polypeptides herein can be incorporated in kits for immunoassays. Pharmaceutical compositions containing the ob polypeptide and antibodies to the ob polypeptide or to the ob receptor can be used for administration to animals and humans.

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Nucleic acids for treating obesity

Field of the Invention

This invention relates to the discovery that an ob polypeptide can be effectively expressed in a host cell using a nucleic acid molecule that places the *ob* gene under regulatory control of an expression control sequence that is not naturally associated with the *ob* gene. Moreover, the recombinantly expressed ob polypeptide is biologically active in inhibiting food intake and/or weight gain. This invention also relates to vectors and host cells comprising the nucleic acid molecule, methods for production of an ob polypeptide, for induction of production of an ob polypeptide, for inhibition of weight gain and/or food intake, for treatment of obesity, for inhibition of the activity of the ob polypeptide, for identification, detection and isolation of an ob receptor, and for identification or detection of an ob polypeptide or a homolog thereof. This invention further relates to an ob polypeptide produced in accordance with the methods as mentioned, antibodies to ob polypeptides, a method for isolation of antibodies to ob receptors, kits comprising such antibodies, and pharmaceutical compositions comprising ob polypeptides.

Background of the Invention

Obesity, the condition of excessive accumulation of fat, or excessive adiposity, affects 30% of the human population and is a serious health hazard. It is generally believed that appetite, energy balance and body weight gain are modulated by diverse neurochemical and neuroendocrine signals from different organs in the body and diverse regions in the brain. The hypothalamus plays an important function in this process, acting through a variety of systems that involve a close interaction between nutrients, amines, neuropeptides and hormones, as noted in Leibowitz, *Trends in Neurosciences* (1992) *15*: 491-497.

Two peptides in the brain have received considerable attention with respect to their role in modulating behavioral and physiological functions essential to nutrient and energy balance. They are neuropeptide Y (NPY), a 36 amino acid peptide member of the pancreatic polypeptide family, and galanin (GAL), a 29 amino acid chain that is amidated

at the COOH terminus. Both peptides are densely concentrated in the brain and particularly in the hypothalamus, which also contains high levels of the mRNA and receptor sites for these peptides as described in Leibowitz, MODELS OF NEUROPEPTIDE ACTION, *Ann. N.Y. Acad. Sci.* (1984) *739*: 12.

In addition, other effectors have been found to have a role in modulating nutrient ingestion and metabolism, including the glucocorticoid corticosterone (CORT), as described in *J. Neuroendocrinology* (1994) *6*: 479-501; the duodenal peptide cholecystokinin (CCK), as described in Smith and Gibbs (1992), MULTIPLE CHOLECYSTOKININ RECEPTORS IN THE CNS (Oxford Univ. Press, Oxford) pp. 166-182; and insulin, as described in Schwartz *et al.* (1994), *Endocrinol. Rev. 2(1):* 109-113.

Other researchers have approached the problem of understanding the regulation of food intake and energy output by genetic analysis of animals carrying mutant genes. The first of the recessive obesity mutations, the *obese* mutation (*ob)* was identified and described in 1950 by Ingall *et al., J. Hered.* (1950) *41* : 317-318. Subsequently, 5 single-gene mutations in mice have been observed to produce an obese phenotype, as described in Friedman *et al.* (1990), *Cell 69*: 217-220. More recently, the mouse *obese* gene and its human homologue have been cloned, as described in Zhang *et al.* (1994), *Nature 372:* 425.

Zhang *et al.* reports the cloning and sequencing of the mouse *ob* gene, which encodes a 4.5 kilobase adipose tissue messenger RNA (mRNA), with a highly conserved 167 amino acid open reading frame. Based upon the nucleotide sequence and the deduced amino acid sequence of the open reading frame, Zhang *et al.* postulates that the product of the *ob* gene is a 167 amino acid ob protein, presumably having a signal peptide at the N-terminus of the ob protein. Zhang *et al.* does not show, however, that the 167 amino acid polypeptide is indeed secreted, or what, if any, part of this polypeptide is actually secreted. Moreover, Zhang *et al.* also does not show that the polypeptide product of the ob gene has any particular bioactivity or function.

Strategies for treatment for obesity in the past have included dieting, surgery (lipectomy), and drug therapies including the insulin normalization drug Ro 23-7637, antilipolytic agents such as SDZ WAG 994 developed at Sandoz Research Institute,

CCK-A agonists such as FPL 15849KF developed at Fisons Pharmaceuticals and those developed at Glaxo Research Institute, the anorectic dexfenfluramine developed at Lederle Laboratories, the serotonin uptake inhibitor, fluoxetine, developed at Lilly Research Laboratories, the drug sibutramine developed at Boots Pharmaceuticals, the anti-diabetic of β-adrenergic receptor agonists developed at Bristol-Myers Squibb and American Cyanamid, and enterostatin and thermogenic agents developed at Amylin Pharmeuticals.

To date, despite these various methods of treatment, long term recovery from the condition of obesity is rare. Further, although the ob protein has been identified in mice and its homologue identified in humans, an exact determination of the amino acid sequence of the mature native ob protein has not been made, nor has the exact sequence of the serum form of the molecule been determined. Notably, the relationship or interaction between the ob protein and other factors that affect behavorial and physiological functions essential to nutrient and energy balance is still very much a mystery.

Summary of the Invention

It is, therefore, an object of the present invention to provide a better understanding of the function of the ob protein and a better understanding of the regulatory mechanism governing food intake and/or energy output.

It is also an object of the present invention to provide a nucleic acid molecule that can be expressed in a cell to provide a polypeptide that can be used for control of obesity and the problems associated with obesity, such as type II diabetes. The polypeptide encoded by such a nucleic acid molecule is referred to herein as the ob polypeptide.

It is another object of the present invention to provide a nucleic acid molecule as above that additionally contains a secretion leader coding sequence to allow secretion of the ob polypeptide from a host cell upon expression.

It is another object of the present invention to provide an expression vector containing such nucleic acid molecules and host cells containing such vectors.

It is also an object of the present invention to provide a method for producing the ob polypeptide, for example, by recombinant DNA techniques.

It is still another object of the present invention to induce the production of the ob polypeptide.

It is yet another object of the present invention to provide a method for inhibition of food intake and/or a method for inhibition of weight gain.

It is also another object of the present invention to provide a method for blocking the activity of the ob polypeptide.

It is also an object of the present invention to provide antibodies to the ob polypeptide, pharmaceutical compositions containing such antibodies for therapeutic purposes, and kits for detection of an ob polypeptide, or a homolog thereof, containing such antibodies.

It is still another object of the present invention to provide methods for isolation, identification, detection, or production of a receptor of the ob polypeptide, that is, an ob receptor, a method for identification and/or detection of an ob polypeptide or a homolog thereof, a method for production of antibodies to the ob receptor.

It is yet another object of the present invention to provide a method for inhibition of the activity of an ob polypeptide.

In accordance thereto, there is provided herein a nucleic acid molecule that contains a first nucleotide sequence that encodes an expression control sequence and a second nucleotide sequence that encodes an ob polypeptide, the second nucleotide sequence being under regulatory control of the first nucleotide sequence, and the first nucleotide sequence is not naturally associated with the second nucleotide sequence.

In accordance to another object of the present invention, there is provided a nucleic acid molecule as above, further containing a third nucleotide sequence, the third nucleotide sequence encoding a secretion leader sequence that is sufficient for secretion of the ob polypeptide upon expression of the nucleic acid molecule in a host cell.

In accordance to another object of the present invention, there is provided herein an expression vector and a host cell containing the expression vector which contains the nucleic acid molecule as above.

In accordance to a further object of the present invention, there is provided a method of production of an ob polypeptide by providing the nucleic acid molecule as

above, introducing the nucleic acid molecule into a cell and allowing expression of the ob polypeptide in the cell.

There is further provided, in accordance to another object of the present invention, a method of production of the ob polypeptide by providing the vector as above, introducing the vector into a host cell, and allowing expression of the ob polypeptide in the cell.

There is also provided herein a method for production of an ob polypeptide by providing a host cell transformed with the vector as above and allowing expression of the ob polypeptide in the cell.

In accordance to still another object of the present invention, there is provided a method for induction of production of an ob polypeptide *in vivo* by administering the nucleic acid molecule as above, either directly or by viral or non-viral means, or by administering the vector as above.

In accordance to yet another object of the present invention, there is provided an ob polypeptide produced by the process of providing a host cell transformed with the nucleic acid molecule or the vector as above, and allowing the expression of the ob polypeptide in the host cell.

In accordance to still another object of the present invention, there is provided a method for inhibition of weight gain and/or a method of inhibition of food intake by administration of a therapeutically effective amount of the ob polypeptide.

In accordance to yet a further object of the present invention, there is provided a method for identification, isolation, detection or production of an ob receptor by providing a labeled ob polypeptide, allowing the labeled ob polypeptide to react with the ob receptor to form a binding pair, and determining the identity of the binding pair, in particular, the identity of the molecule binding to the labeled ob polypeptide.

In accordance to still another object of the present invention, there is provided an antibody to the ob polypeptide as above and a method of producing an antibody to an ob receptor, the antibody to the ob polypeptide being capable of forming a specific binding pair with the ob polypeptide, and the antibody to the ob receptor being capable of forming a specific binding pair with the ob receptor.

In accordance to another object of the present invention, there is provided a method for blocking the activity of the ob polypeptide with the use of inhibitors thereto, such as antibodies to the ob polypeptide.

In accordance to a further object of the present invention, there is provided a method for identification or detection of an ob polypeptide or a homolog thereof, involving contacting an antibody directed to an ob polypeptide as above with a sample suspected of containing an ob polypeptide or a homolog thereof, allowing the mixture to react to form a specific binding pair, and determining the presence of a specific binding pair, the antibody being labeled with a detectable marker to facilitate easy identification or detection.

There is further provided, in accordance with another object of the present invention, a kit for detection of an ob polypeptide or a homolog thereof, and a kit for detection of antibodies to the ob polypeptide, the kits containing either labeled antibodies or labeled ob polypeptides.

There is also provided, in accordance with a further object of the present invention, a pharmaceutical composition containing the ob polypeptide and a pharmaceutically acceptable carrier and a pharmaceutical composition containing antibodies to the ob polypeptide and a pharmaceutically acceptable carrier.

Further objects, features, and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description, while indicating preferred embodiments of the present invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows diagramatically the DNA constructs each comprising a nucleotide sequence that encodes an ob polypeptide. The numbers on the left: #1122, #1123, #1124, #1132, #1130, #1131, #1119, #1129, #1127, #1150 and #1128 are the designated constructs number. The term "CMV" indicates that the promoter in the construct is

derived from cytomegalovirus ("CMV"). The term "T7" indicates that the promoter in those constructs is a T7 promoter.

Figure 2 shows diagrammatically other DNA constructs each comprising a nucleotide sequence that encodes an ob polypeptide. The numbers on the left: #1144, #1142, #1143, #1145, and #1147 are the designated constructs number. The term "SRα" indicates the source of the promoter (SV40/HIV hybrid promoter). Constructs #1145 and #1147 each comprises viral sequences from Moloney murine leukemia virus.

Figure 3 reflects the effect of intravenous administration of the ob protein expressed from construct #1127 on the weight of treated CD rats as compared to that of the untreated controls.

Figure 4 shows the amount of food consumption by the CD rats treated with ob protein as compared to that of untreated controls.

Figure 5 shows the weight of the fecal matter excreted by CD rats treated with ob protein as compared to that of untreated controls.

Figure 6 shows the urine output of CD rats treated with ob protein as compared to that of untreated controls.

Figure 7 shows the amount of water intake by CD rats treated with ob protein as compared to that of untreated controls.

Figures 8a and 8b show the argument map of DNA construct #1122.

Figure 9 shows the nucleotide sequences of DNA constructs #1130, #1131 and #1132, respectively, and alignments of these sequences.

Figure 10 shows the argument map of DNA construct #1119.

Figure 11 shows the argument map of DNA construct #1127.

Figure 12 shows the argument map of DNA construct #1150.

Detailed Description of the Preferred Embodiments

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All published work, including patents, and patent applications cited herein are hereby incorporated by reference.

The inventors herein have discovered that a nucleic acid molecule encoding an ob polypeptide can be expressed in a recombinant expression system to produce the ob polypeptide. The nucleic acid molecule can also be used in the context of an expression vector that contains one or more expression control sequences for expression of the ob polypeptide in a host cell. Moreover, the nucleic acid molecule can be used for gene therapy purposes for production or induction of production of the ob polypeptide, for example, in *ex vivo* or *in vivo* gene therapy, the nucleic acid molecule to be delivered either directly or by viral or non-viral means.

The ob polypeptide produced herein is useful for inhibition of food intake, and/or inhibition of weight gain and, in essence, in the treatment of obesity or consequences of obesity including type II diabetes. The ob polypeptide can, furthermore, be used for production of monoclonal or polyclonal antibodies which, in turn, can be used, for example, in immunoassays for detection or identification of an ob polypeptide or a homolog thereof. Inhibitors to the ob polypeptide, such as antibodies, can be used to block the activity of the ob polypeptide. Such blocking activity is useful, for example, for stimulating appetite in subjects suffering from poor food intake and/or poor nutrition resulting from, for example, diseases or chronic conditions such as anorexia nervosa, psychiatric conditions, or during recovery from surgery. The antibodies to the ob polypeptide, therefore, can be incorporated into a kit that can contain other conventional reagents for immunoassays or into a pharmaceutical composition for therapeutic administration. The ob polypeptide can also be labeled with an identifiable marker, such as a radioactive marker, and be used to detect the presence of an ob receptor that specifically binds the ob polypeptide, forming a binding pair. Pharmaceutical compositions containing the ob polypeptide can also be made for administration and treatment.

The ob receptor identified in this manner can be sequenced, and used to make a probe for probing a cDNA library to obtain a coding sequence. This ob receptor coding sequence can then be used to make the ob receptor recombinantly in the same manner as for the ob polypeptide. Polyclonal and monoclonal antibodies to the ob receptor can be made which can also be used in a kit for detection or identification of an ob receptor.

Notably, the inventors herein have found that a mature ob polypeptide can be effectively expressed using recombinant DNA technology and that after purification or partial purification, the expressed ob polypeptide has biological activity. Such biological activity includes the ability to inhibit weight gain and/or food intake. No toxic effects of the administration of the ob protein have been observed.

The present invention may be better understood in light of the following definitions incorporated herein.

Definitions

A "nucleic acid molecule" or a "coding sequence," as used herein, refers to either RNA or DNA that encodes a specific amino acid sequence or its complementary strand.

The term "an expression control sequence" refers to a sequence that is conventionally used to effect expression of a gene that encodes a polypeptide and include one or more components that affect expression, including transcription and translation signals. Such a sequence includes, for example, one or more of the following: a promoter sequence, an enhancer sequence, an upstream activation sequence, a downstream termination sequence, a polyadenylation sequence, an optimal 5' leader sequence to optimize initiation of translation in mammalian cells, and a Shine-Dalgarno sequence. The expression control sequence that is appropriate for expression of the present polypeptide differs depending upon the host system in which the polypeptide is to be expressed. For example, in prokaryotes, such a control sequence can include one or more of a promoter sequence, a ribosomal binding site, and a transcription termination sequence. In eukaryotes, for example, such a sequence can include a promoter sequence, and a transcription termination sequence. If any necessary component of an expression control sequence is lacking in the nucleic acid molecule of the present invention, such a component can be supplied by the expression vector to effect expression. Expression control sequences suitable for use herein may be derived from a prokaryotic source, an eukaryotic source, a virus or viral vector or from a linear or circular plasmid. Further details regarding expression control sequences are provided below.

The term "ob protein" refers to the putative murine polypeptide containing a sequence of 167 amino acid residues predicted from the isolated cDNA encoding the *ob*

gene, as well as its homolog in mammalian and non-mammalian species, as described in Zhang *et al.*, mentioned above.

Á "mature ob protein" refers to the ob protein as above except that it lacks the putative signal peptide sequence.

The term "ob polypeptide" includes the ob protein and the mature ob protein as defined above and further include truncations, variants, allelles, analogs and derivatives thereof. Unless specifically mentioned otherwise, such ob polypeptides possess one or more of the bioactivities of the ob protein, such as those discovered herein. This term is not limited to a specific length of the product of the *ob* gene. Thus, polypeptides that are identical or contain at least 60%, preferably 70%, more preferably 80%, and most preferably 90% homology to the *ob* protein or the mature ob protein, wherever derived, from human or nonhuman sources are included within this definition of the ob polypeptide. Also included, therefore, are alleles and variants of the product of the *ob* gene that contain amino acid substitutions, deletions, or insertions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acid residues such as to alter a glycosylation site, a phosphorylation site, an acetylation site, or to alter the folding pattern by altering the position of the cysteine residue that is not necessary for function, etc. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted, for example, substitutions between the members of the following groups are conservative substitutions: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, and Phe/Trp/Tyr. Analogs include peptides having one or more peptide mimics, also known as peptoids, that possess ob protein-like activity. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and nonnaturally occurring. The term "ob polypeptide" also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like.

The term "leader sequence" refers to either a translated amino acid sequence situated 5' to the N-terminus of a polypeptide sequence to be expressed, or an

untranslated nucleotide sequence. This term includes at least one of the following and can be a combination thereof: a secretion leader sequence, as defined below, a fusion protein leader sequence, and an untranslated nucleotide sequence. The translated amino acid leader sequence can be used herein to optimize secretion, as in a secretion leader sequence. Alternatively, the translated amino acid leader sequence can be used to optimize initiation of translation, as by the use of all or part of a tk leader sequence. Moreover, the amino acid sequence used to optimize initiation of translation can be used in combination with a secretion leader sequence. A fusion protein leader sequence can be used to optimize intracellular production of a polypeptide such as an ubiquitin/ob polypeptide fusion protein for intracellular expression in yeast. A 5' untranslated leader sequence can be used to optimize transcription, if desired.

The term "secretion leader sequence" refers to a polypeptide that, when encoded at the N-terminus of a protein, causes the protein to be secreted from the site of synthesis, typically the endoplasmic reticulum, to another location, such as the periplasmic space in prokaryotes or extracellularly into the culture medium in which the host is being propagated. The secretion leader sequence can be a signal peptide sequence or can include other sequences that include glycosylation sites or processing sites for production of a mature protein. Such sequences can be derived from any source that is suitable for expression in the desired host or can be hybrid sequences or synthetic sequences. For example, suitable secretion leaders for use in yeast include the *Saccharomyces cerevisiae* α-factor leader (U.S. Pat. No. 4,870,008), a-factor leader sequence, truncated α-factor leader sequence, yeast killer toxin leader sequence, and α-amylase or glucoamylase leader sequence. Hybrid leader sequence can include, for example, a signal peptide sequence linked to a processing site for production of a mature polypeptide upon cleavage at the processing site, for example, a yeast invertase signal sequence can be used in combination with a KEX 2 cleavage site (Lys-Arg) to produce a hybrid leader sequence. Moreover, random peptide sequences for any host expression system such as those generated by a combinatorial library can be screened for ones that are useful as leader sequences for a desired host. Bacterial leader sequences useful herein are ones that lead to the production of a polypeptide that is secreted into the periplasmic space. For example, β-lactamase signal peptide sequence. Mammalian leader sequences include leader sequences of

proteins that are normally secreted into the serum including, for example, proteins such as albumin, immunoglobulin, Factor VII, secreted hormones, blood-borne factors such as insulin, growth factors or can be sequences derived from fat cells. The following genes also have leader sequences that facilitate secretion from mammalian cells and can be used for the secretion of heterologous proteins in mammalian cell systems: human influenza virus A, human preproinsulin, and bovine growth hormone, among others. Further details regarding secretion leader sequences are provided below.

The term "weight gain inhibitory amount" refers to that amount that is effective for production of inhibition of weight gain of an individual. The precise inhibitory amount varies depending upon the health and physical condition of the individual to be treated, the capacity of the individual's ability to adjust to the change in metabolism and body size, the formulation, and the attending physician's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The term "food intake inhibitory amount" refers to that amount that is effective for production of inhibition of food intake of an individual. The precise inhibitory amount varies depending upon the health and physical condition of the individual to be treated, the capacity of the individual to adjust to the inhibition of food intake, the formulation, and the attending physician's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

A "therapeutically effective amount" is generically that amount that will generate the desired therapeutic outcome and includes, for example, a "weight gain inhibitory amount" and a "food intake inhibitory amount."

The term "ob receptor" refers to a structure, generally a protein, located on or in a cell membrane that specifically recognizes a sequence of amino acids of the ob polypeptide so as to bind to it with a higher affinity than to a random polypeptide, forming a binding pair. Such an interaction between the ob polypeptide and the ob receptor is expected to trigger an intracellular response.

The term "binding pair" refers to a pair of molecules, usually referring to a protein/protein pair, but does not exclude a protein/DNA pair, in which the components

of the pair bind specifically to each other with a higher affinity than to a random molecule, such that upon binding, for example, in case of a ligand/receptor interaction, the binding pair triggers a cellular or an intracellular response. An example of a ligand/receptor binding pair is a pair formed between PDGF (platelet derived growth factor) and a PDGF receptor. An example of a different binding pair is an antigen/antibody pair in which the antibody is generated by immunization of a host with the antigen. Specific binding indicates a binding interaction having a low dissociation constant, which distinguishes specific binding from non-specific, background, binding.

The term "kit" refers to a package containing the specified material and includes printed instructions for use of the material. For example, the kit can be an immunoassay kit containing antibodies to detect an antigen, such as an ob polypeptide or an ob receptor, or it can be an assay kit containing antigens to detect antibodies. "Printed instructions" may be written or printed on paper or other media, or committed to electronic media such as magnetic tape, computer-readable disks or tape, CD-ROM, and the like. Kits may also include plates, tubes, dishes, diluents, solvents, wash fluid or other conventional reagents.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or an ob polypeptide, *in vivo,* and refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying

agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

Although the methodology described below is believed to contain suffcient details to enable one skilled in the art to practice the present invention, other constructs not specifically exemplified, such as plasmids, can be constructed and purified using standard recombinant DNA techniques described in, for example, Sambrook *et al.* (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2d edition (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), under the current regulations described in United States Dept. of HEW, NATIONAL INSTITUTE OF HEALTH (NIH) GUIDELINES FOR RECOMBINANT DNA RESEARCH. These references include procedures for the following standard methods: cloning procedures with plasmids, transformation of host cells, plasmid DNA purification, phenol extraction of DNA, ethanol precipitation of DNA, agarose gel electrophoresis, purification of DNA fragments from agarose gels, and restriction endonuclease and other DNA-modifying enzyme reactions.

The coding sequence of the ob protein for purposes herein can be obtained based on the DNA sequence of the ob protein disclosed in Zhang *et al.*, cited bove, using any number of conventional techniques, such as polymerase chain reaction ("PCR"). One example of such a technique is the reverse transcription PCR ("RT-PCR"). Under this methodology, poly A+ RNA can be isolated from adipose tissue and reversed transcribed to produce a first strand cDNA, using a reverse primer and reverse transcriptase. The reverse primer contains nucleotides of a portion of the noncoding strand of the *ob* gene. For example, suitable for use herein is reverse primer #553 containing nucleotides 593-616 of the non-coding strand extended with nucleotides of a linker. This PCR reaction mixture can then be used for amplification using the same reverse primer and a forward primer containing nucleotides from a portion of the coding strand of the ob gene. For example, forward primer #552, containing nucleotides 115-134 of the coding strand extended with nucleotides for a linker can be used herein. The amplified DNA can be

purified and used as a template for generation of other ob DNA constructs for protein expression in prokaryotes and eukaryotes.

Variations of this ob construct can be made by conventional techniques, including PCR or site directed mutagenesis. These variants can be made to create, a truncated protein, such as an ob protein minus its signal sequence, to insert restriction sites or linker sequences, and to add a tag to facilitate detection of the construct, for example, addition of a Myc or HA (influenza virus hemagglutinin) sequence which can be detected by use of anti-Myc or anti-HA antibodies, respectively.

As an example, a truncated ob polypeptide lacking amino acids 1-21, the presumed signal sequence, can be made by use of the full-length cDNA construct, a forward primer such as primer #560 containing nucleotides 178-197 of the coding strand and a linker sequence, and the reverse primer #558, mentioned above. An initiator codon for expression in prokaryotes can be added in the form of a linker.

DNA constructs containing the tags for identification purposes can be synthesized using PCR. For example, DNA construct #1150 encodes an ob polypeptide that contains an epitope tag for purposes of antibody recognition. This construct can be made using the full length ob DNA construct, a forward primer, such as primer #560, and a reverse primer, such as #559 containing nucleotides 602 to 616 of the noncoding strand, extended with a *Sma*I restriction site, and amplifying the DNA by PCR using standard protocol. The amplified DNA fragment can then be ligated into a vector that contains a sequence coding for heart muscle kinase and the Myc epitope, for example.

The DNA construct made as above described can be ligated to an expression plasmid containing an appropriate promoter for expression in a desired host expression system. Expression plasmids with various promoters are currently available commercially. For example, the plasmid pET23 can be purchased from Novagen (Madison, WI). This plasmid utilizes a T7 promoter sequence for expression in bacteria. Commercially available mammalian expression plasmids can also be used for the present purposes. In the present instance, the plasmid pCG used herein is obtained and is available from Qianjin Hu at the University of California, San Francisco, CA. This plasmid is a derivative of pEVRF, and directs expression in mammalian cells from the human CMV

promoter/enhancer region. Further details regarding expression systems are provided below.

Expression in Bacterial Cells

Bacterial expression systems can be used with the present constructs. Control elements for use in bacteria include promoters, optionally containing operator sequences, and ribosome binding sites. Useful promoters include sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the β-lactamase (*bla*) promoter system, bacteriophage λPL, and T7. In addition, synthetic promoters can be used, such as the *tac* promoter. The β-lactamase and lactose promoter systems are described in Chang *et al., Nature* (1978) *275*: 615, and Goeddel *et al., Nature* (1979) *281*: 544; the alkaline phosphatase, tryptophan (trp) promoter system are described in Goeddel *et al., Nucleic Acids Res.* (1980) *8*: 4057 and EP 36,776 and hybrid promoters such as the *tac* promoter is described in U.S. Patent No. 4,551,433 and deBoer *et al., Proc. Natl. Acad. Sci. USA* (1983) *80*: 21-25. However, other known bacterial promoters useful for expression of eukaryotic proteins are also suitable. A person skilled in the art would be able to operably ligate such promoters to the present ob coding sequences, for example, as described in Siebenlist *et al., Cell* (1980) *20*: 269, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (SD) sequence operably linked to the DNA encoding the target polypeptide. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence can be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat stable enterotoxin II leaders. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

The foregoing systems are particularly compatible with *Escherichia coli*. However, numerous other systems for use in bacterial hosts including Gram-negative or Gram-positive organisms such as *Bacillus spp., Streptococcus spp., Streptomyces spp., Pseudomonas* species such as *P. aeruginosa, Salmonella typhimurium,* or *Serratia*

marcescans, among others. Methods for introducing exogenous DNA into these hosts typically include the use of $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation, nuclear injection, or protoplast fusion as described generally in Sambrook *et al.* (1989), cited above. These examples are illustrative rather than limiting. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media, as described generally in Sambrook *et al.*, cited above.

Expression in yeast cells

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, among others, the following yeasts: *Saccharomyces cerevisiae* ,as described in Hinnen *et al., Proc. Natl. Acad. Sci. USA* (1978) *75*: 1929; Ito *et al., J. Bacteriol.* (1983) *153*: 163; *Candida albicans* as described in Kurtz *et al., Mol. Cell. Biol.* (1986) *6*: 142; *Candida maltosa*, as described in Kunze *et al., J. Basic Microbiol.* (1985) *25*: 141; *Hansenula polymorpha*, as described in Gleeson *et al., J. Gen. Microbiol.* (1986) *132*: 3459 and Roggenkamp *et al., Mol. Gen. Genet.* (1986) *202* :302); *Kluyveromyces fragilis*, as described in Das *et al., J. Bacteriol.* (1984) *158*: 1165; *Kluyveromyces lactis*, as described in De Louvencourt *et al., J. Bacteriol.* (1983) *154*: 737 and Van den Berg *et al., Bio/Technology* (1990) *8*: 135; *Pichia guillerimondii*, as described in Kunze *et al., J. Basic Microbiol.* (1985) *25*: 141; *Pichia pastoris*, as described in Cregg *et al., Mol. Cell. Biol.* (1985) *5*: 3376 and U.S. Patent Nos. 4,837,148 and 4,929,555; *Schizosaccharomyces pombe*, as described in Beach and Nurse, *Nature* (1981) *300*: 706; and *Yarrowia lipolytica*, as described in Davidow *et al., Curr. Genet.* (1985) *10*: 380 and Gaillardin *et al., Curr. Genet.* (1985) *10*: 49, *Aspergillus* hosts such as A. *nidulans*, as described in Ballance *et al., Biochem. Biophys. Res. Commun.* (1983) *112*: 284-289; Tilburn *et al., Gene* (1983) *26*: 205-221 and Yelton *et al., Proc. Natl. Acad. Sci. USA* (1984) *81*: 1470-1474, and *A. niger*, as described in Kelly and Hynes, *EMBO J.* (1985) *4*: 475479; *Trichoderma reesia*, as described in EP 244,234, and filamentous fungi such as, e.g, *Neurospora, Penicillium, Tolypocladium*, as described in WO 91/00357.

Control sequences for yeast vectors are known and include promoters regions from genes such as alcohol dehydrogenase (ADH), as described in EP 284,044, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK), as described in EP 329,203. The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences, as described in Myanohara *et al., Proc. Natl. Acad. Sci. USA* (1983) *80*: 1. Other suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase, as described in Hitzeman *et al., J. Biol. Chem.* (1980) *255*: 2073, or other glycolytic enzymes, such as pyruvate decarboxylase, triosephosphate isomerase, and phosphoglucose isomerase, as described in Hess *et al., J. Adv. Enzyme Reg.* (1968) *7*: 149 and Holland *et al., Biochemistry* (1978) *17*: 4900. Inducible yeast promoters having the additional advantage of transcription controlled by growth conditions, include from the list above and others the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP 073,657. Yeast enhancers also are advantageously used with yeast promoters. In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, upstream activating sequences (UAS) of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region, as described in U.S. Patent Nos. 4,876,197 and 4,880,734. Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, or *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as *GAP* or *PyK*, as described in EP 164,556. Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription.

Other control elements which may be included in the yeast expression vectors are terminators, for example, from *GAPDH* and from the enolase gene, as described in Holland *et al., J. Biol. Chem.* (1981) *256*: 1385, and leader sequences which encode signal sequences for secretion. DNA encoding suitable signal sequences can be derived from genes for secreted yeast

proteins, such as the yeast invertase gene as described in EP 012,873 and JP 62,096,086 and the α-factor gene, as described in U.S. Patent Nos. 4,588,684, 4,546,083 and 4,870,008; EP 324,274; and WO 89/02463. Alternatively, leaders of non-yeast origin, such as an interferon leader, also provide for secretion in yeast, as described in EP 060,057.

Methods of introducing exogenous DNA into yeast hosts are well known in the art, and typically include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations.

Transformations into yeast can be carried out according to the method described in Van Solingen *et al., J. Bact.* (1977) *130*: 946 and Hsiao *et al., Proc. Natl. Acad. Sci. USA* (1979) *76*: 3829. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used as described generally in Sambrook *et al.*, cited above.

For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, α-factor, or acid phosphatase leaders. The origin of replication from the 2μ plasmid origin is suitable for yeast. A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid described in Kingsman *et al., Gene* (1979) *7*: 141 or Tschemper *et al., Gene* (1980) *10*: 157. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* Gene.

For intracellular production of the present polypeptides in yeast, a sequence encoding a yeast protein can be linked to a coding sequence of the ob polypeptide to produce a fusion protein that can be cleaved intracellularly by the yeast cells upon expression. An example, of such a yeast leader sequence is the yeast ubiquitin gene.

Expression in Insect Cells

Baculovirus expression vectors (BEVs) are recombinant insect viruses in which the coding sequence for a foreign gene to be expressed is inserted behind a baculovirus promoter in place of a viral gene, e.g., polyhedrin, as described in Smith and Summers, U.S. Pat. No., 4,745,051.

An expression construct herein includes a DNA vector useful as an intermediate for the infection or transformation of an insect cell system, the vector generally containing DNA coding for a baculovirus transcriptional promoter, optionally but preferably, followed downstream by an insect signal DNA sequence capable of directing secretion of a desired protein, and a site for insertion of the foreign gene encoding the foreign protein, the signal DNA sequence and the foreign gene being placed under the transcriptional control of a baculovirus promoter, the foreign gene herein being the coding sequence of the ob polypeptide.

The promoter for use herein can be a baculovirus transcriptional promoter region derived from any of the over 500 baculoviruses generally infecting insects, such as, for example, the Orders Lepidoptera, Diptera, Orthoptera, Coleoptera and Hymenoptera including, for example, but not limited to the viral DNAs *of Autographo californica* MNPV, *Bombyx mori* NPV, *rrichoplusia ni* MNPV, *Rachlplusia ou* MNPV or *Galleria mellonella* MNPV, *Aedes aegypti, Drosophila melanogaster, Spodoptera frugiperda,* and *Trichoplusia ni*. Thus, the baculovirus transcriptional promoter can be, for example, a baculovirus immediate-early gene IEI or IEN promoter; an immediate-early gene in combination with a baculovirus delayed-early gene promoter region selected from the group consisting of a 39K and a *Hin*dIII fragment containing a delayed-early gene; or a baculovirus late gene promoter. The immediate-early or delayed-early promoters can be enhanced with transcriptional enhancer elements.

Particularly suitable for use herein is the strong polyhedrin promoter of the baculovirus, which directs a high level of expression of a DNA insert, as described in Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W.Doerfler, ed.); EP 127,839 and EP 155,476; and the promoter from the gene encoding the p10 protein, as described in Vlak *et al., J. Gen. Virol.* (1988) *69*: 765-776.

The plasmid for use herein usually also contains the polyhedrin polyadenylation signal, as described in Miller *et al., Ann. Rev. Microbiol.* (1988) *42*: 177 and a procaryotic ampicillin-resistance (*amp*) gene and an origin of replication for selection and propagation in *E. coli*. DNA encoding suitable signal sequences can also be included and is generally derived from genes for secreted insect or baculovirus proteins, such as the

baculovirus polyhedrin gene, as described in Carbonell *et al.*, *Gene* (1988) *73*: 409, as well as mammalian signal sequences such as those derived from genes encoding human α-interferon as described in Maeda *et al.*, *Nature* (1985) *315*: 592-594; human gastrin-releasing peptide, as described in Lebacq-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) *8*: 3129; human IL-2, as described in Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) *82*: 8404; mouse IL-3, as described in Miyajima *et al.*, *Gene* (1987) *58*: 273; and human glucocerebrosidase, as described in Martin *et al.*, *DNA* (1988) *7*:99.

Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified and can be used herein. See, for example, the description in Luckow *et al.*, *Bio/Technologvy* (1988) *6:* 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) *315*: 592-594. A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV. Such viruses may be used as the virus for transfection of host cells such as *Spodoptera frugiperda* cells.

Other baculovirus genes in addition to the polyhedrin promoter may be employed to advantage in a baculovirus expression system. These include immediate-early (alpha), delayed-early (beta), late (gamma), or very late (delta), according to the phase of the viral infection during which they are expressed. The expression of these genes occurs sequentially, probably as the result of a "cascade" mechanism of transcriptional regulation. Thus, the immediate-early genes are expressed immediately after infection, in the absence of other viral functions, and one or more of the resulting gene products induces transcription of the delayed-early genes. Some delayed-early gene products, in turn, induce transcription of late genes, and finally, the very late genes are expressed under the control of previously expressed gene products from one or more of the earlier classes. One relatively well defined component of this regulatory cascade is IEI, a preferred immediate-early gene of *Autographo californica* nuclear polyhedrosis virus (AcMNPV). IEI is pressed in the absence of other viral functions and encodes a product that stimulates the transcnption of several genes of the delayed-early class, including the preferred 39K gene, as described in Guarino and Summers, *J. Virol.* (1986) *57*: 563-571 and *J. Virol.* (1987) *61*:

2091-2099 as well as late genes, as described in Guanno and Summers, *Virol.* (1988) *162*: 444-451.

Immediate-early genes as described above can be used in combination with a baculovirus gene promoter region of the delayed-early category. Unlike the immediate-early genes, such delayed-early genes require the presence of other viral genes or gene products such as those of the immediate-early genes. The combination of immediate-early genes can be made with any of several delayed-early gene promoter regions such as 39K or one of the delayed-early gene promoters found on the *Hin*dIII fragment of the baculovirus genome. In the present instance, the 39 K promoter region can be linked to the foreign gene to be expressed such that expression can be further controlled by the presence of IEI, as described in L. A. Guarino and Summers (1986a), cited above; Guarino & Summers (1986b) *J. Virol.*, (1986) *60*: 215-223, and Guarino *et al.* (1986c), *J. Virol.* (1986) *60*: 224-229.

Additionally, when a combination of immediate-early genes with a delayed-early gene promoter region is used, enhancement of the expression of heterologous genes can be realized by the prescence of an enhancer sequence in direct cis linkage with the delayed-early gene promoter region. Such enhancer sequences are characterized by their enhancement of delayed-early gene expression in situations where the immediate-early gene or its product is limited. For example, the hr5 enhancer sequence can be linked directly, in cis, to the delayed-early gene promoter region, 39K, thereby enhancing the expression of the cloned heterologous DNA as described in Guarino and Summers (1986a), (1986b), and Guarino *et al.* (1986).

The polyhedrin gene is classified as a very late gene. Therefore, transcription from the polyhedrin promoter requires the previous expression of an unknown, but probably large number of other viral and cellular gene products. Because of this delayed expression of the polyhedrin promoter, state-of-the-art BEVs, such as the exemplary BEV system described by Smith and Summers in, for example, U.S. Pat. No., 4,745,051 will express foreign genes only as a result of gene expression from the rest of the viral genome, and only after the viral infection is well underway. This represents a limitation to the use of existing BEVs. The ability of the host cell to process newly synthesized proteins decreases as the baculovirus infection progresses. Thus, gene expression from the polyhedrin promoter occurs at a time when the host cell's ability to process newly synthesized proteins is potentially diminished for certain proteins such as human tissue plasminogen activator. As a consequence, the expression of secretory glycoproteins in BEV

systems is complicated due to incomplete secretion of the cloned gene product, thereby trapping the cloned gene product within the cell in an incompletely processed form.

While it has been recognized that an insect signal sequence can be used to express a foreign protein that can be cleaved to produce a mature protein, the present invention is preferably practiced with a mammalian signal sequence for example the ob signal sequence.

An exemplary insect signal sequence suitable herein is the sequence encoding for a Lepidopteran adipokinetic hormone (AKH) peptide. The AKH family consists of short blocked neuropeptides that regulate energy substrate mobilization and metabolism in insects. In a preferred embodiment, a DNA sequence coding for a Lepidopteran *Manduca sexta* AKH signal peptide can be used. Other insect AKH signal peptides, such as those from the Orthoptera *Schistocerca gregaria* locus can also be employed to advantage. Another exemplary insect signal sequence is the sequence coding for Drosophila cuticle proteins such as CPI, CP2, CP3 or CP4.

Currently, the most commonly used transfer vector that can be used herein for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, can also be used herein. Materials and methods for baculovirus/insect cell expression systems are commercially available in a kit form from companies such as Invitrogen (San Diego CA) ("MaxBac" kit). The techniques utilized herein are generally known to those skilled in the art and are fully described in Summers and Smith, A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experiment Station Bulletin No. 1555, Texas A&M University (1987); Smith *et al., Mol. Cell. Biol.* (1983) *3*: 2156, and Luckow and Summers (1989). These include, for example, the use of pVL985 which alters the polyhedrin start codon from ATG to ATT, and which introduces a *Bam*HI cloning site 32 basepairs downstream from the ATT, as described in Luckow and Summers, *Virology* (1989) *17*:31.

Thus, for example, for insect cell expression of the present polypeptides, the desired DNA sequence can be inserted into the transfer vector, using known techniques. An insect cell host can be cotransformed with the transfer vector containing the inserted desired DNA together with the genomic DNA of wild type baculovirus, usually by cotransfection. The vector and viral genome are allowed to recombine resulting in a recombinant virus that can be easily identified and purified.

The packaged recombinant virus can be used to infect insect host cells to express the ob polypeptide.

Other methods that are applicable herein are the standard methods of insect cell culture, cotransfection and preparation of plasmids are set forth in Summers and Smith (1987), cited above. This reference also pertains to the standard methods of cloning genes into AcMNPV transfer vectors, plasmid DNA isolation, transferring genes into the AcmMNPV genome, viral DNA purification, radiolabeling recombinant proteins and preparation of insect cell culture media. The procedure for the cultivation of viruses and cells are described in Volkman and Summers, *J. Virol.* (1975) *19*: 820-832 and Volkman, *al., J. Virol.* (1976) *19*: 820-832.

Expression in Mammalian Cells

The ob polypeptides of the present invention can be expressed in mammalian cells, such as adipocytes, or fat cells, using promoters and enhancers that are functional in the such cells. For example, the 422(aP2) gene and the stearoyl-CoA desaturase 1 (SCD1) gene contain suitable adipocyte-specific promoters, as described in Christy *et al., Genes Dev.* (1989) *3*:1323-1335. Synthetic non-natural promoters or hybrid promoters can also be used herein. For example, a T7T7/T7OB promoter can be constructed and used, in accordance with Chen *et al., Nucleic Acids Res. 22:* 2114-2120 (1994), where the T7 polymerase is under the regulatory control of its own promoter and drives the transcription of the ob coding sequence, which is placed under the control of another T7 promoter. The primary determinant for the fat-specific expression is an enhancer located at about > 5 kb upstream of the transcriptional start site, as described in Ross *et al., Proc. Natl. Acad. Sci.USA* (1990) *87*:9590-9594 and Graves *et al., Genes Dev.* (1991) *5*:428-437. Also suitable for use herein is the gene for the CCAAT/enhancer-binding protein C/EBPα, which is highly expressed when 3T3-L1 adioblast commit to the differentiation pathway and in mature post-mitotic adipocytes, as described in Birkenmeier *et al., Genes Dev.* (1989) *3*:1146-1156. The recently isolated transcription factor PPARγ2, expressed exclusively in adipocyte tissues, as described in Tontonoz *et al., Cell* (1994) *79*:1147-1156, can also be used herein.

Typical promoters for mammalian cell expression include the SV40 early promoter, the CMV promoter, the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other non-viral promoters, such as a promoter derived from the murine metallothionein gene, will also find use in mammalian constructs. Mammalian expression may be either constitutive or regulated (inducible), depending on the promoter. Typically, transcription termination and polyadenylation sequences will also be present, located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the ob polypeptide coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those derived from SV40, as described in Sambrook et al. (1989), cited previously. Introns, containing splice donor and acceptor sites, may also be designed into the constructs of the present invention.

Enhancer elements can also be used herein to increase expression levels of the mammalian constructs. Examples include the SV40 early gene enhancer, as described in Dijkema *et al.*, *EMBO J.* (1985) *4*: 761 and the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* (1982b) *79*: 6777 and human cytomegalovirus, as described in Boshart *et al.*, *Cell* (1985) *41*: 521. A leader sequence can also be present which includes a sequence encoding a signal peptide, to provide for the secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the gene of interest such that the leader sequence can be cleaved either *in vivo* or *in vitro*. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

There exist expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Once complete, the mammalian expression vectors can be used to transform any of several mammalian cells. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216.

Mammalian cell lines available as hosts for expression are also known and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), human embryonic kidney cells, baby hamster kidney cells, mouse sertoli cells, canine kidney cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, as well as others.

The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* (1979) *58*: 44, Barnes and Sato, *Anal. Biochem.* (1980) *102*: 255, U.S. Patent Nos. 4,767,704, 4,657,866, 4,927,762, or 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors such as insulin, transferrin, or epidermal growth factor, salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin(tm) M drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Gene Therapy Applications

The nucleic acid constructs that contain the ob polypeptide coding sequence ("*ob* coding sequence"), with or without the signal sequence, can be used for inhibition of food intake and weight gain , such as for treatment of obesity or the problems associated with obesity, by administration thereof via gene therapy. Gene therapy strategies for delivery of such constructs can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence *in vivo* can be either constitutive or regulated.

For delivery using viral vectors, any of a number of viral vectors can be used, as described in Jolly, *Cancer Gene Therapy 1:* 51-64 (1994). For example, the *ob* coding sequence can beinserted into plasmids designed for expression in retroviral vectors, as described in Kimura *et al., Human Gene Therapy* (1994) *5:* 845-852, adenoviral vectors, as described in Connelly *et al., Human Gene Therapy* (1995) *6:* 185-193, adeno-associated viral vectors, as described in Kaplitt *et al., Nature Genetics* (1994) *6*: 148-153 and sindbis vectors. Promoters that are suitable for use with these vectors include the Moloney retroviral LTR, CMV promoter and the mouse albumin promoter. Replication competent free virus can be produced and injected directly into the animal or humans or by transduction of an autologous cell *ex vivo,* followed by injection *in vivo* as described in Zatloukal *et al., Proc. Natl. Acad. Sci. USA* (1994) *91:* 5148-5152.

The *ob* coding sequence can also be inserted into plasmid for expression of the ob polypeptide *in vivo* or *ex vivo*. For *in vivo* therapy, the coding sequence can be delivered by direct injection into tissue or by intravenous infusion. Promoters suitable for use in this manner include endogenous and heterologous promoters such as CMV. Further, a synthetic T7T7/T7OB promoter can be constructed in accordance with Chen *et al.* (1994), *Nucleic Acids Res. 22:* 2114-2120, where the T7 polymerase is under the regulatory control of its own promoter and drives the transcription of the ob coding sequence, which is also placed under the control of a T7 promoter. The coding sequence can be injected in a formulation comprising a buffer that can stablize the coding sequence

and facilitate transduction thereof into cells and/or provide targeting, as described in Zhu *et al., Science* (1993) *261:* 209-211.

Expression of the *ob* coding sequence *in vivo* upon delivery for gene therapy purposes by either viral or non-viral vectors can be regulated for maximal efficacy and safety by use of regulated gene expression promoters as described in Gossen *et al., Proc. Natl. Acad. Sci. USA* (1992) *89:*5547-5551. For example, the *ob* coding sequence can be regulated by tetracycline responsive promoters. These promoters can be regulated in a positive or negative fashion by treatment with the regulator molecule.

For non-viral delivery of the *ob* coding sequence, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, *J. Biol. Chem.* (1987) *262:* 4429-4432; insulin, as described in Hucked *et al., Biochem. Pharmacol. 40:* 253-263 (1990); galactose, as described in Plank *et al., Bioconjugate Chem. 3:*533-539 (1992); lactose, as described in Midoux *et al., Nucleic Acids Res. 21:* 871-878 (1993); or transferrin, as described in Wagner *et al., Proc. Natl. Acad. Sci. USA 87:*3410-3414 (1990). Other delivery systems include the use of liposomes to encapsulate DNA comprising the *ob* gene under the control of a variety of tissue-specific or ubiquitously-active promoters, as described in Nabel *et al., Proc. Natl. Acad. Sci. USA 90:* 11307-11311 (1993), and Philip *et al., Mol. Cell Biol. 14:* 2411-2418 (1994). Further non-viral delivery suitable for use includes mechanical delivery systems such as the biolistic approach, as described in Woffendin *et al., Proc. Natl. Acad. Sci. USA* (1994) *91(24):* 11581-11585. Moreover, the ob coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the *ob* coding sequence include, for example, use of hand held gene transfer particle gun, as described in U.S. 5,149,655; use of ionizing radiation for activating transferred gene, as described in U.S. 5,206,152 and PCT application WO 92/11033.

Upon expression of the ob polypeptide *in vitro* in any of the above-described expression systems, and after recovery and, optionally, folding, and purification of the ob

polypeptide, in accordance with conventional methods, the ob polypeptide can be used in a variety of ways. For example, the ob polypeptide can be administered intravenously, subcutaneously, peritoneally, intramuscularly, or orally in a therapeutically effective amount for inhibition of weight gain and/or inhibition of food intake. The specific amount to be given is a weight-gain inhibitory amount or a food-intake inhibitory amount as previously defined. Preferably, the ob polypeptide is administered intravenously or subcutaneously in the form of a pharmaceutical composition which, preferably, contains a pharmaceutically acceptable carrier.

Furthermore, the ob protein, with or without its natural secretion leader sequence, can be used to identify an ob receptor, having specific affinity for the ob protein. For this purpose, the ob protein can be labeled with a conventional marker, such as a radioactive label, and the labeled ob protein is allowed to react with cells, cell extracts, or cell membranes belonging to one or more cell types. The mixture is then examined for presence of specific binding to the labeled ob protein. The binding pairs formed can be separated by conventional techniques, such as by use of solvents or denaturing reagent or by passage through a column that selectively bind one member of the pair, and eluting the opposite member, i.e., the ob receptor, with an appropriate solvent.

The ob receptor can be purified by conventional techniques and the amino acid sequence thereof determined. Based upon the amino acid sequence identified, an oligonucleotide probe can be made to probe a cDNA or genomic DNA library. Clones that hybridize to the probe can be amplified and sequenced. A cDNA clone that encodes a full length ob receptor can be used for recombinant production of large quantities of the receptor, useful for further studies into the mechanism of of regulation of obesity and to obtain agonists and antagonists thereto.

The ob polypeptide and the ob receptor of the present invention can further be used to generate monoclonal or polyclonal antibodies. Antibodies to the proteins of the invention, both polyclonal and monoclonal, may be prepared by conventional methods. In general, the protein is first used to immunize a suitable animal, preferably a mouse, rat, rabbit or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally performed by mixing or emulsifying the protein in

saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 μg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation, for example, at about 1,000 x g for 10 minutes. About 20-50 ml per bleed may be obtained from rabbits.

Monoclonal antibodies (MAbs) are prepared using the method of Kohler and Milstein, *Nature* (1975) *256*: 495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen, and optionally several large lymph nodes, is removed and dissociated into single cells. If desired, the spleen cells may be screened, after removal of nonspecifically adherent cells, by applying a cell suspension to a plate or well coated with the protein antigen. B-cells expressing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium such as one containing, e.g., hypoxanthine, aminopterin, and thymidine (a "HAT" medium). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen, and which do not bind to unrelated antigens. The selected MAb-secreting hybridomas are then cultured either *in vitro*, e.g., in tissue culture bottles or hollow fiber reactors, or *in vivo*, as ascites in mice.

If desired, the antibodies, whether polyclonal or monoclonal, may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase (HRP) is usually detected by its ability to convert

3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a MAb. Further, one may combine various labels for desired effect. For example, MAbs and avidin also require labels in the practice of this invention: thus, one might label a MAb with biotin, and detect its presence with avidin labeled with ^{125}I, or with an anti-biotin MAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

The antibodies generated in this manner can be used in any conventional applications, including for diagnostic and therapeutic purposes. For example, as a diagnostic, it can be used in an immunoassay for identification or detection of an ob polypeptide or a homolog thereof in a sample suspected of containing such. For this purpose, the antibodies can be labeled with a suitable marker, such as a radioactive label, and allowed to react with the sample. After an appropriate length of time, the sample can be examined for the presence of specific binding pairs. Presence of specific binding suggests that an ob polypeptide or a homolog thereof is present in the sample.

The antibodies to the ob polypeptide, polyclonal or monoclonal, and preferably monoclonal, can be used for therapeutic purposes for blocking the in vivo activity of the ob polypeptide. Such antibodies will be compatible to the host to be treated. For example, for treatment of humans, the antibodies can be human monoclonal antibodies or humanized antibodies, as the term is generally known in the art. The humanized antibodies can be made by any number of conventional methods. For example, by cdr (complementarity determining region) grafting, veneering, phage libray display, or by use of xeno-mouse. In cdr grafting, the coding regions of the cdr of murine antibodies are linked to the coding regions of the framework regions of human antibodies. In veneering,

the canonical regions of the antibodies, including parts of the cdr and parts of the murine framework regions that are exposed on the surface of the molecule, are maintained as well as the murine cdr regions. The antibodies to be administered can be given in a therapeutically effective amount, and can be in the form of a pharmaceutical composition.

Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. The assays herein involve the use of labeled antibodies to the ob polypeptide or labeled ob polypeptides. The labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe can alsobe used; examples of which are assays which utilize biotin and avidin, and enzyme labeled and mediated immunoassays, such as ELISA assays.

The enzyme-linked immunosorbent assay (ELISA) can be used, for example, to measure either antigen, the ob polypeptide, concentration or the antibody to the ob polypeptide concentration. This method depends upon conjugation of an enzyme to either the antigen or to the antibody, and uses the bound enzyme activity as a quantitative label. To measure the antibody concentration, the antigen is fixed to a solid phase, such as a microplate or plastic cup, incubated with dilutions of the sample to be tested. The mixture is then washed, incubated with anti-immunoglobulin labeled with an enzyme, and washed again. Enzymes suitable for labeling are known in the art, and include, for example, horseradish peroxidase. Enzyme activity bound to the solid phase is measured by adding the specific substrate, and determining product formation or substrate utilization colorimetrically. The enzyme activity bound is a direct function of the amount of antibody bound.

To measure antigen concentration, that is, ob polypeptide concentration, a known specific antibody is fixed to the solid phase, the test material containing antigen is added. After incubation, the solid phase is washed, and a second enzyme-labeled antibody is added. After washing, substrate is added, and enzyme activity is estimated colorimetrically, and related to antigen concentration. Immunofluorescence assays can

also be performed with such antibodies and antigens, as described in Hashido *et al.*, *Biochem. Biophys. Res. Comm.* (1992) *187(3):* 1241-1248.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the polypeptides of the present invention or antibodies directed against the ob polypeptides in suitable containers, along with the remaining reagents and materials required for the conduct of the assay, as well as a suitable set of assay instructions. The other materials or reagents include, for example, diluents, wash and other reagents, appropriate containers such as tubes, plates, etc.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Example 1

For easy reference, the nucleic acid constructs made herein and as shown in FIG. 1 and FIG. 2 are summarized below. In addition to the summary, further details regarding the constuction of the nucleic acid molecules follow.

Construct #1122, as shown in FIG. 1, is a schematic representation of an ob coding region in pCG expression plasmid. Numbers on top indicate amino acid positions in the ob protein. The letters "MASR" indicate four additional amino acids, represented by its one letter code, fused to the ob sequence by virtue of the cloning procedure used. These amino acids are part of the upstream *tk* leader region and ensure optimized initiation of translation of the ob mRNA. The ob coding region is flanked by restriction enzyme recognition sequences for *Xba*I and *Bam*HI. The bar in the box represents the putative signal sequence cleavage site.

Constructs #1123 and #1124, as shown in FIG. 1, are schematic representations similar to that of construct #1122 except that the ob stop codon was removed and replaced with a linker comprising a *Sma*I recognition sequence followed by nucleotide sequences for either the Myc or the HA epitope.

Constructs #1130-1132, as shown in FIG. 1, are schematic representations of the ob coding region lacking the putative signal sequence. The ob coding regions encoding amino acids 21 to 167, 22 to 167, or 25 to 167 are each preceded by sequence encoding five additional amino acids "MASRH," each represented by its one-letter code, again due to the construction method used, as indicated above. In addition, the stop codons in these constructs were removed and replaced with a linker comprising a *Sma*I recognition sequence, followed by nucleotide sequences for the Myc epitope. Construct #1132 contains a conservative substitution of leucine to valine at amino acid position 111.

Construct #1119, as shown in FIG. 1, is a schematic representation of the ob coding region that includes a mutation at amino acid 59 generated by the TGA nucleotide sequence. This construct is not utilized for expression of a biologically active ob polypeptide but, instead, is utilized for control and comparison purposes to mimic an obese mutation. An *Nde*I recognition sequence is located at the N-terminus of this ob polypeptide, linking the T7 promoter and the signal sequence of the molecule. A *Bam*HI site is located at the C-terminus of the molecule.

Constructs #1127, #1128, and #1129, as shown in FIG. 1, are schematic representations of the ob coding region carrying truncations of codons for 21, 24, and 20 amino acids, respectively, at the N-termini of the ob polypeptide. These constructs were made in view of the uncertainty of the length of the signal sequence. The construct #1127 was used to produce the ob polypeptide for the experiment involving injection of the ob polypeptide into rats.

Construct #1150, as shown in FIG. 1, is a schematic representation of the ob coding region encoding amino acid 22 to 167 of the ob protein. In addition, the coding region is extended with nucleotide sequences encoding the recognition sequence for heart muscle kinase followed by the Myc epitope sequence.

Constructs #1142, #1143, and #1144, as shown in FIG. 2, are a schematic representation of all or part of the ob coding region linked to a hybrid promoter, SRα. #1144 encodes the full-length ob protein with an *Xba*I site at the N terminus, and a *Bam*HI site at the C terminus. Construct #1142 encodes the full-length ob protein with an *Xba*I site at the N terminus, a *Sma*I site as well as a Myc tag at the C terminus. Construct #1143 encodes a truncated form of the ob protein, lacking amino acids 1-21 at

the N-terminus and contains a *Sma*I site as well as a Myc tag at the C-terminus. The length of the polypeptides encoded by construct #1142 and construct #1143 was tested by transfecting construct #1142 containing the signal sequence and #1143 lacking the signal sequence into mammalian cells (COS cells). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Ob polypeptides were detected using monoclonal Myc antibodies. Two signals were obtained for construct #1142, one corresponding to the precursor form of ob protein and a faster migrating band which co-migrated with the product from construct #1143. The expression product of each of constructs #1123 and #1124 was similarly tested to determine the size of the polypeptides formed. Based on the comigration of the processed forms with the product of #1143, it is reasonable to conclude that the signal sequence consists of 21 amino acids.

Cloning of the Mouse ob Gene

The mouse *ob* gene was cloned by reverse-transcription PCR ("RT-PCR") as follows. RNA was isolated from mouse adipose tissue and polyA+ mRNA was isolated using oligo-dT beads purchased from Dynabead (Dynal A.S., Norway). To synthesize the first strand cDNA, 1 μg of this polyA+ mRNA was reverse transcribed using 1 μg of reverse primer #553, having a sequence as follows:

5'- GCGGATCCTCATGCGCATTCAGGGCTAACATCCAACT-3'. This reverse primer contained nucleotides 593 to 616 of the noncoding strand, as indicated in the underlined portion of the sequence above, extended with a *Bam*HI restriction site, as indicated in the non-underlined portion. Added thereto were 10 Units Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Boehringer Mannheim, Germany), and 25 μM dNTPs. The reaction mixture was incubated at 42°C for 60 minutes. Three μl of the reverse transcriptase reaction were used for PCR amplification using the above mentioned reverse primer and forward primer # 552 having a sequence as follows:

5'-CGCATATGTGCTGGAGACCCCTGT-3'. This forward primer contained nucleotides 115 to 134 of the coding strand, as indicated in the underlined portion of the sequence above, beginning with an *Nde*I restriction site, as indicated in the non-underlined portion of the sequence. The first codon of the ob mRNA, the methionine codon ATG, is part of the *Nde*I restriction site.

The amplified DNA was gel purified and used as a template for generation of various ob constructs for protein expression in prokaryotes and eukaryotes, as exemplified below. The ob expression constructs in FIG. 1 and FIG. 2 show either full-length ob protein or truncated versions thereof lacking variously 1-20, 1-21, or 1-24 of the N-terminal amino acid residues of the ob protein. In addition, in some of the constructs, the ob coding region was fused at the C-terminus to additional nucleotide sequences comprising epitope such as Myc or HA (influenza virus hemagglutinin) for recognition with anti-Myc or anti-HA antibodies or for labeling with heart muscle kinase in the presence of radioactive gamma-[^{32}P]ATP.

Example 2

Construction of the ob Expression Plasmids for Expression of the ob Protein in Bacterial and Mammalian Cells

Additional DNA constructs were made that encoded the full length ob protein as well as truncated forms of it lacking a portion of the N-terminal region or signal sequence of the ob protein were made. Construct #1138 (not shown) was made as follows: The coding sequence of the full length ob protein consisting of amino acids 1 through 167 was synthesized by PCR, using (1) the above-described ob cDNA, (2) forward primer #557 having a sequence: 5'-GCTCTAGAATGTGCTGGAGACCCCTGTG-3', which contained nucleotides 115 to 135 of the coding strand, as indicated by the underlined portion of the above sequence, and beginning with an *Xba*I restriction site, as indicated by the non-underlined portion of the sequence, and (3) reverse primer #558 having a sequence: 5'-GCGGATCCTCAGCATTCAGGGCTAAC-3', which contained nucleotides 602 to 616 of the noncoding strand, as indicated by the underlined portion, and extended with a *Bam*HI restriction site, as indicated by the non-underlined portion. The the full length coding sequence was amplified by PCR using a standard PCR protocol.

The amplified DNA product was ligated into expression plasmid pET23a from Novagen (Madison, WI). This plasmid contains a T7 promoter sequence, for expression in bacteria. In this construct #1138, the expression of the ob protein is under the control of the T7 polymerase which directs transcription from the T7 promoter.

For expression in eukaryotes, construct #1122 was made, essentially in the same way as for construct #1138, except that the DNA fragment encoding the full length ob protein was ligated into eukaryotic expression plasmid pCG. Plasmid pCG, which is a pEVRF derivative, as described in Matthias *et al., Nucleic Acids Res.* (1989) *17*: 6418, has a modified polylinker, and directs expression in mammalian cells from the human cytomegalovirus promoter/enhancer region. In this construct, translation initiation is controlled by the 5' untranslated region of the herpes simplex virus thymidine kinase gene.

Truncated ob polypeptides lacking a portion of the N-terminal region of the ob protein were made. The constructs included are those containing nucleotide sequences encoding amino acids 21 to 167 (construct #1129), 22 to 167 (constructs #1127 and #1150), or 25 to 167 (construct #1128). Construct #1127 was synthesized by PCR using (1) the above described full-length ob cDNA, (2) forward primer #560 containing a sequence as follows:

5'-GCTCTAGACATATGGTGCCTATCCAGAAAGTCC-3'. This primer contained nucleotides 178 to 197 of the coding strand, as indicated by the underlined portion of the sequence, beginning with *Xba*I and *Nde*I restriction sites and (3) reverse primer #558, as described above. This primer contained nucleotides 602 to 616 of the noncoding strand, as indicated by the underlined portion, and extended with a SmaI restriction site, as indicated by the non-underlined portion. The DNA was amplified by PCR using a standard PCR protocol.

The amplified DNA fragment of construct #1127 was ligated into T7 expression plasmid, pHB40P. The initiator methionine for expression in prokaryotes was provided by the *Nde*I restriction site and was not part of the native protein. Vector pHB40P is a derivative of pET plasmid described in Studier *et al., Methods in Enzymol.* (1990) *185*: 60, and contains a different polylinker compared to the pET vector. Expression of the ob polypeptides in this construct is under control of the T7 polymerase which directs transcription from the T7 promoter.

For expression in mammalian cells, the DNA fragment encoding the truncated form of the ob protein was ligated into the eukaryotic expression plasmid pCG as described above for the expression of the full length form of the protein.

Constructs to provide for expression of the ob polypeptides with either an HA or Myc epitope tag were made as follows. Expression construct #1150 identified in FIG. 1 was synthesized by PCR using (1) the above described full length ob cDNA, (2) forward primer #560, described above, and (3) reverse primer #559 containing a sequence as follows: 5'-CGCCCGGGGCATTCAGGGCTAAC-3'. The DNA was amplified by PCR using a standard PCR protocol.

For expression in prokaryotes, the amplified DNA fragment was ligated into vector pHB40P, described above, containing the sequence for heart muscle kinase and the Myc epitope, 5'-CCCGGGGGAC GCAGAGCTTC CGTGGAGCAG AAGCTGATTT CCGAGGGAGG ACCTGAACTGA. The final construct is identified in FIG. 1 as #1150.

Constructs #1123 and #1124, as indicated in FIG. 1, were synthesized by PCR using (1) the above-described full length ob cDNA, (2) forward primer #557, described above, and (3) reverse primer #559, described above. For expression in mammalian cells, the DNA fragments were ligated into a pCG vector, described above, containing either the epitope sequence for the monoclonal antibody against HA or Myc. The final constructs # 1123 and #1124 are identified in FIG. 1.

Constructs #1142, #1143, and #1144 were made for expression in eukaryotes, and contained no additional amino acids from the vector, as shown in FIG. 2. The nucleotide sequence encoding the full-length ob protein (construct #1144) and versions lacking the putative signal sequence without additional amino acid residues were constructed in plasmid pBJ-1. Plasmid pBJ-1 is a pcDL-SRα296 derivative, as described in Takebe *et al., Mol. Cell. Biol.* (1988) *8*: 466-472, with a modified polylinker, and directs expression in mammalian cells from the SRα promoter. SRα is composed of the simian virus 40 (SV40) early promoter and the R segment and part of the U5 sequence (R-U5') of the long terminal repeat (LTR) of human T-cell leukemia virus type 1. Construct #1144 and #1142 represent the full-length ob protein with and without the Myc tag, respectively. Construct #1143 represents ob protein lacking the first 21 amino acids. This construct also contains the Myc tag. Constructs #1142 and #1143 further comprises a *Sma*I restriction site 5' to the Myc epitope tag.

Constructs #1147 and #1145, identified in FIG. 2, contained the coding sequence of the full-length ob protein, with or without Myc epitope tag, respectively, inserted into

the retrovirus vector pBabePuro. This vector was described in Morgenstein and Land, *Nucleic Acids Res.* (1990) *12*: 3587-3596. Expression of the ob polypeptide with the pBabePuro vector is under the control of the M-MuLV long terminal repeat and can be additionally regulated with a T7 promoter. The "Puro" sequence encoding puromycin resistance is under the regulatory control of a SV40 promoter.

Example 3

Expression of the ob Protein in Bacteria; Isolation of the ob Protein and Generation of Polyclonal Antibodies Against the ob Protein

The ob DNA constructs containing the T7 promoter, including the DNA encoding the full length and the truncated forms of the ob protein, were transformed, respectively into *E. coli* strain BL21 (pLysS), which was described in Studier *et al., Methods in Enzymol.* (1990) *185*: 60, for expression. Overexpressed ob protein was isolated from inclusion bodies by dissolving the cell pellet in phosphate-saline buffer containing about 5 mM PMSF (phenylmethylsulfonyl fluoride) and about 2 mM DTT. The suspension was sonicated, adjusted to a final concentration of about 0.5 M NaCl and 1.0% Triton-X-100. The lysate was cleared by centrifugation at about 12 K rpm for about 10 minutes at 4°C. The pellet was washed with phosphate-saline buffer to remove traces of Triton-X-100. Inclusion bodies were dissolved in about 7 M guanidium-hydrochloride in about 100 mM phosphate buffer and 10 mM Tris-HCl (pH 7.5). Insoluble particles were removed by centrifugation. Solubilized ob polypeptides were dialyzed against 7 M urea containing about 10 mM NaCl and 20 mM Tris-HCl (pH 7.5) in several steps. The ob polypeptides were purified by ion-exchange chromatography using Q-sepharose purchased from Pharmacia (Piscataway, N.J.) For refolding, the ob polypeptides were dialyzed against phosphate-saline buffer containing about 1 M NaCl at a concentration of about 10 to about 150 µg/ml. After dialysis, against only phosphate-saline buffer, the protein was concentrated to about 1 mg/ml.

Substantially purified ob polypeptides were used to raise polyclonal antibodies in rabbits using standard techniques. Such polyclonal antibodies were raised by E.L Labs (Soquel, CA) in accordance with the following protocol: Three animals were immunized by intramuscular injections into the hind leg at two sites and by subcutaneous injection at

the scruff of the back. This mode of immunization has been approved by the NIH. The inocula consisted of the antigen, the ob protein, in 0.5 ml saline and an equal volume of adjuvant. Complete Freund's adjuvant was used for the first immunization and incomplete Freund's adjuvant was used for all other boosts. Animals were immunized at approximately 3 week intervals. Test bleeds were performed via the central ear artery, and samples of about 5 to 10 ml are collected. Test bleeds were taken once as a prebleed, and then at weeks 4, 5 and 7 after immunization. Animals were exsanguinated once the titer in the sera was acceptable.

Example 4

Expression of the ob Protein in Insect Cells

For expression of the ob protein in insect cells, the wild-type mouse ob sequence was excised from the pCG vector by cutting with *Xba*I and *Bam*HI. This insert was then cloned into the PAcC13 vector using the *Xba*I and *Bam*HI sites. This construct was transfected into SF9 cells using the following procedure. The ob coding sequence was recombined into the *Autographa californica* baculovirus, AcNPV, via the pAcC13 transfer vector, as described in Munesmitsu *et al.*, *Mol. Cell. Biol.* (1990) *10*: 5977-5982, by co-transfecting about 2 μg of transfer vector with about 0.5 μg of linearized, wild-type viral DNA into SF9 cells as described in Kitts *et al.*, *Nucleic Acids Res.* (1990) *18*: 5667-5672. Recombinant baculovirus was isolated by plaque purification as described in Smith *et al.*, *Mol. Cell. Biol.* (1983) *3*: 2156-2165. Suspension cultures of about 1.5 x 10^6 SF9 cells per ml were harvested for ligan binding following about 48 hours infection with the relevant baculovirus at m.o.i. (multiplicity of infection) of about 2-10, in serum-free medium, as described in Malorella *et al.*, *Biotechnol.* (1988) *6*: 1406-1510.

After about 48 hours, secretion of ob polypeptide into the culture medium of the SF9 was observed. The ob polypeptide was visualized by Coomassie stain and also by Western analysis using ob antibody. A two-step purification procedure was employed. First the protein was purified via DEAE anion exchange chromatography. The protein fractions were pooled and applied to a Sephacryl 100 column and eluted with phosphate buffered saline (PBS). The ob-containing fractions were visualized by staining with Coomassie. The ob protein was purified as one product. A slightly faster migrating

species was seen on sodium dodecyl sulfate (SDS) gel electrophoresis in the absence of dithiothreitol. This most likely confirmed the presence of one disulfide bond in the ob polypeptide.

Example 5

In Vitro Kinase Reaction of the ob Protein Tagged with Heart Muscle Kinase Recognition Sequence and Detection of the ob Protein in Eukaryotic Cells

Heart muscle kinase tagged ob polypeptide was purified from bacteria, as described above. Approximately 2 μg of purified ob polypeptide was labeled in about 20 μl of 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 12 mM MgCl2 and 1 mM DTT with about 100 μCi [γ 32P]ATP purchased from Amersham, which had an activity of about >6000 Ci/mMol, together with about 10 Units of heart muscle kinase from Sigma. The solution was incubated for about 30 minutes at 37°C. Labeled ob polypeptide was separated from unincorporated [γ 32P]ATP by ion exchange chromatography, as described above using Q-sepharose.

This labeled ob polypeptide is used to react with cells, cell extracts, cell membranes or portions thereof to look for specific binding of a receptor to the labeled ob polypeptide. The specific binding pair obtained in this manner is separated from the reaction mix and is dissociated by conventional techniques. The putative receptor member of the binding pair is then characterized, for example, to determine molecular size, and all or a portion of the amino acid sequence. Based upon the information obtained from amino acid sequence analysis, the coding sequence of the ob receptor is cloned, in accordance with conventional techniques. Thus, an oligonucleotide probe is constructed and labeled with an identifiable marker, such as a radioactive label. This labeled oligonucleotide probe is then used to probe a cDNA or genomic library to look for specific hybridization. A clone signifying the presence of a full length coding region for an ob receptor is then isolated and its nucleotide sequence determined. This clone is used for recombinant production of the ob receptor.

The ob constructs containing the CMV promoter/enhancer were transiently transformed into COS-7 cells. For immunoblotting, cell extracts were boiled in sample buffer, as described in Laemmli *et al.*, *Nature* (1970) 227: 680, separated by SDS-PAGE

and transferred to nitrocellulose filters. Filters were blocked in TBST buffer consisting of about 10 mM Tris-HCl (pH 7.5), 130 mM NaCl, 0.05% Tween20, and 0.2% sodium azide containing about 3% non-fat dried milk. The filters were incubated with polyclonal antibodies against the ob polypeptide or monoclonal antibodies directed against epitope tagged ob proteins. Bound antibodies were detected with an alkaline phosphatase conjugated anti-rabbit or anti-mouse antibody from Promega.

Example 6

Intravenous Administration of the ob Protein in CD Rats

The bacterially expressed and purified ob polypeptide of the construct #1127, identified in FIG. 1, was administered to each of 3 CD rats. Three other CD rats were not treated but serve as controls. The rats were all male rates about 7 weeks old. All of them were housed in metabolic cages for monitoring body weight, food consumption, fecal weight, water intake, and urine output. Urinalysis was done daily to determine protein, glucose, ketones, nitrite, uribilinogen, bilirubin, blood, pH, and leukocyte levels.

More specifically the animals were housed in metabolic cages for 24 hours before administration of the ob polypeptide. The polypeptide was administered twice a day daily for 4 days (BID X4) at about 1 mg/kg per day by jugular cannulae. Food was removed for 2-3 hours before dosing and for 1-2 hours after dosing. Body weight, food consumption, fecal weight, water intake, and urine output were monitored daily. Urinalysis was done daily to determine the protein, glucose, ketones, nitrite, urobilinogen, bilirubin, blood, pH, and leukocyte levels.

The results of this study are reflected in the graphs in FIGs. 3-7. FIG. 3 is a graph of the weight of treated CD rats. FIG. 3 shows that upon administration of the ob polypeptide, expressed by the DNA construct #1127, the treated rats, treated on days 1, 2, 3, 4, and 5, showed inhibition of weight gain during the 5 days of treatment. Thereafter, the weight gained by the treated animals paralleled that gained by the control animals during an observation period of up to day 16. This result suggests that a periodic dosing regimen, such as weekly or bimonthly, consisting of 4 to 5 days of administration of the ob polypeptide at each dosing, may be effective in maintaining a low weight-gain profile.

FIG. 4 shows the amount of food consumed in grams by the treated and untreated rats. Results show that consumption of food in the ob-treated rats was inhibited as compared to the untreated controls.

FIG. 5 shows the weight of fecal matter excreted by the ob-treated and untreated controls. Results show that the fecal weight from ob-treated rats is reduced as compared to the untreated controls. In general, the graphs of the fecal weight versus days show the same general pattern compared to the graphs of food consumption versus days.

FIG. 6 shows no statistical significance in urine output between the ob-treated rats and the untreated rats.

FIG. 7 shows the water intake pattern of ob-treated and untreated rats.

These results reflect that the CD rats that were admininstered ob protein did not gain weight, and ob protein appears to reduce the animals' food intake and fecal output; water intake of the ob treated rats was slightly depressed and the urinary output appeared normal. The control rats gained weight, and had higher food intake and fecal weights than the ob-treated rats.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

Deposition of Cultures

The constructs made herein that encode the full-length ob polypeptide and truncated versions thereof in plasmids pCG, pET23a, and pBabePuro vector have been deposited at the A.T.C.C. (Parklawn Drive, Rockville, MD, U.S.A.).

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a first nucleotide sequence that encodes an expression control sequence and a second nucleotide sequence that encodes an ob polypeptide, wherein the second nucleotide sequence is under regulatory control of the first nucleotide sequence, and the first nucleotide sequence is not naturally associated with the second nucleotide sequence.

2. The nucleic acid molecule of claim 1, further comprising a third nucleotide sequence, wherein the third nucleotide sequence encodes a secretion leader sequence that is sufficient for secretion of the ob polypeptide upon expression of the nucleic acid molecule in a host cell.

3. The nucleic acid molecule of claim 2, wherein the secretion leader sequence is not naturally associated with the ob polypeptide.

4. The nucleic acid molecule of claim 1, wherein the expression control sequence is one selected from the group consisting of a prokaryotic cell promoter and an eukaryotic cell promoter or a viral promoter.

5. An expression vector comprising the nucleic acid molecule of claim 1 and a third nucleotide sequence that encodes a marker.

6. The vector of claim 5, further comprising a fourth nucleotide sequence, wherein the fourth nucleotide sequence encodes a secretion leader sequence that is sufficient for secretion of the ob polypeptide upon expression of the nucleic acid molecule in a host cell.

7. The vector of claim 6, wherein the secretion leader sequence is not naturally associated with the ob polypeptide.

8. The vector of claim 5, wherein the vector comprises a nucleic acid molecule selected from the group consisting of constructs #1122, #1123, #1124, #1130, #1131, #1132, #1127, #1128, #1129, #1150, #1142, #1143, #1144, #1145, and #1147.

9. A host cell comprising the vector of claim 5.

10. The host cell of claim 9, wherein the vector further comprises a third nucleotide sequence, wherein the third nucleotide sequence encodes a secretion leader sequence that is sufficient for secretion of the ob polypeptide upon expression of the nucleic acid molecule in a host cell.

11. The host cell of claim 9, wherein the cell is selected from a group consisting of a prokaryotic cell and an eukaryotic cell.

12. The host cell of claim 9, wherein the cell is a prokaryotic cell, and prokaryotic cell is *Escherichia coli*.

13. The host cell of claim 11, wherein the cell is an eukaryotic cell, and the eukaryotic cell is selected from the group consisting of a mammalian cell, an insect cell, and a yeast cell.

14. The host cell of claim 13, wherein the eukaryotic cell a mammalian cell and the mammalian cell is a human cell.

15. A method for the production of an ob polypeptide comprising:

a) providing the nucleic acid molecule of claim 1;

b) introducing the nucleic acid molecule into a cell that is capable of expressing the ob polypeptide; and

c) allowing the expression of the ob polypeptide in the cell.

16. A method for production of an ob polypeptide comprising:

a) providing the vector of claim 5;

b) introducing the vector into a host cell; and

c) allowing expression of the ob polypeptide in the cell.

17. A method for production of an ob polypeptide comprising:

a) providing the host cell of claim 9; and

b) allowing expression of the ob polypeptide in the cell.

18. A method for induction of production of an ob polypeptide in a mammal comprising administering to a mammal the nucleic acid molecule of claim 1.

19. The method of claim 18, wherein the nucleic acid molecule is delivered directly or by viral or non-viral means.

20. A method for induction of production of an ob polypeptide in a mammal comprising administering to the mammal the vector of claim 5.

21. An ob polypeptide produced by the method comprising:

a) providing a host cell comprising the nucleic acid molecule of claim 1; and

b) allowing the expression of the ob polypeptide.

22. A method for inhibition of weight gain comprising administering a weight-gain-inhibitory amount of the ob polypeptide of claim 21.

23. A method for the inhibition of food intake comprising administering a food-intake-inhibitory amount of the ob polypeptide of claim 21.

24. A method for production of an ob receptor comprising:

a) providing a labeled ob polypeptide, wherein the ob polypeptide that is labeled is the polypeptide of claim 21;

b) allowing the labeled ob polypeptide to react with cells or portions or extracts thereof to form a binding pair; and

c) separating from the binding pair an ob receptor that binds to the labeled ob polypeptide.

25. An antibody comprising an amino acid sequence, wherein the sequence is capable of binding to the ob polypeptide of claim 21 to form a binding pair.

26. The antibody of claim 25, wherein the antibody is a mammalian antibody or a humanized antibody.

27. The antibody of claim 26, wherein the antibody is a murine antibody or a human antibody.

28. A method for identification of an ob polypeptide or an ob polypeptide homolog comprising contacting a labeled antibody with a sample suspected of containing an ob polypeptide or an ob polypeptide homolog to allow formation of a binding pair, and determining the identity of the binding pair, wherein the antibody that is labeled is the antibody of claim 25.

29. A method for production of an antibody to an ob receptor comprising administering to an animal the ob receptor of claim 30, and collecting from the animals either serum containing antibodies or spleen cells for the production of monoclonal antibodies.

30. An ob receptor produced by the method of claim 24.

31. A method for detection of an ob receptor comprising:

a) providing a labeled antibody to an ob receptor, wherein antibody that is labeled is the antibody of claim 29;

b) allowing the labeled antibody to react with a cell or portions or extracts thereof to allow formation of a binding pair; and

c) determining the presence of the binding pair.

32. A method for the detection of an ob polypeptide comprising:

a) providing a labeled antibody to an ob polypeptide, wherein the antibody that is labeled is the antibody of claim 25;

b) allowing the labeled antibody to react with an ob polypeptide to form a binding pair; and

c) determining the presence of the binding pair.

33. A kit for detection of an ob polypeptide comprising the antibody of claim 25.

34. A kit for detection of an ob receptor comprising the antibody of claim 29.

35. A kit for the detection of an ob receptor comprising a labeled ob polypeptide, wherein the polypeptide that is labeled is the polypeptide of claim 21.

36. A kit for the detection of antibodies to an ob polypeptide comprising a labeled ob polypeptide, wherein the polypeptide that is labeled is the polypeptide of claim 21.

37. A pharmaceutical compositon comprising the ob polypeptide of claim 21 and a pharmaceutically acceptable carrier.

38. A method of blocking the activity of ob polypeptide in a mammal by administering the antibody of claim 25.

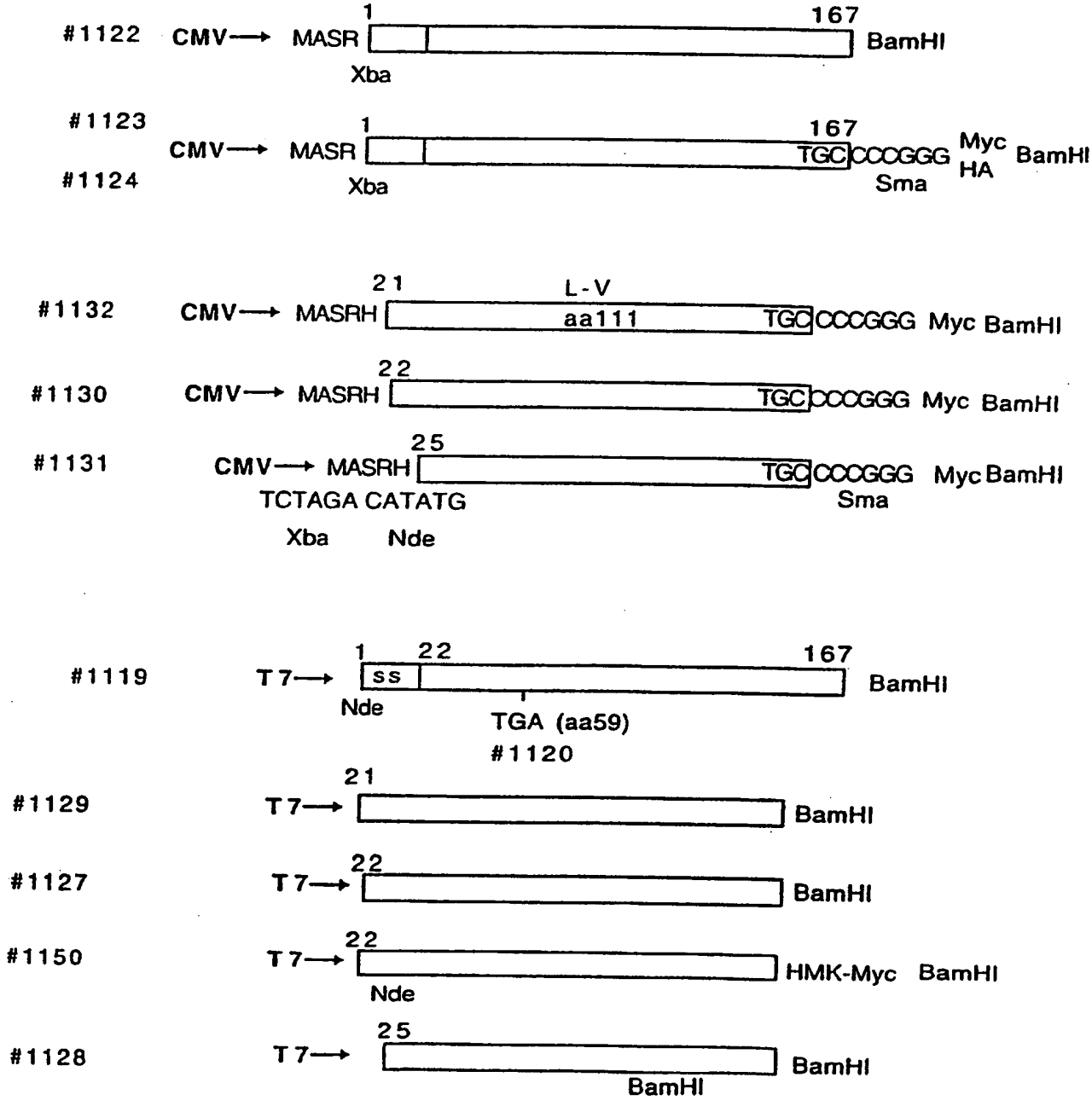


FIG. 1

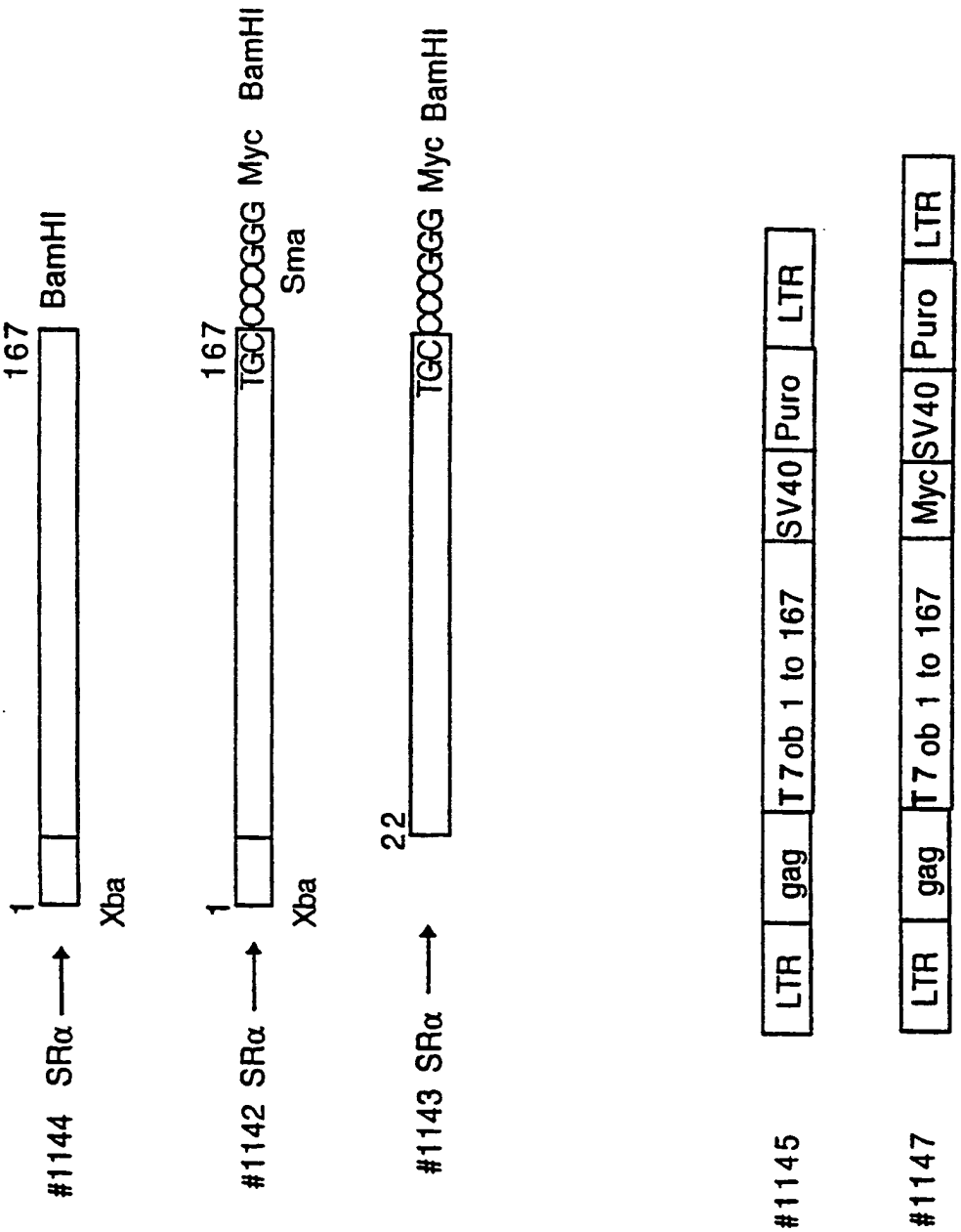


FIG. 2

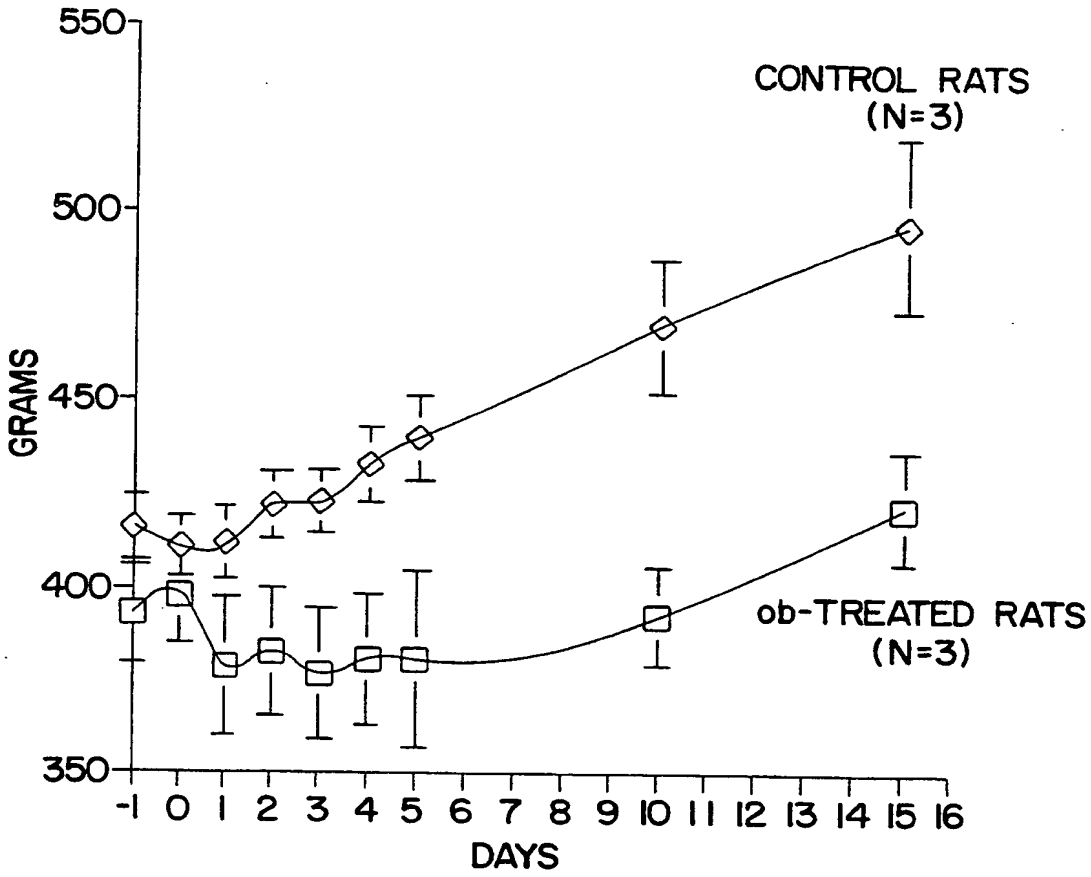


FIG. 3

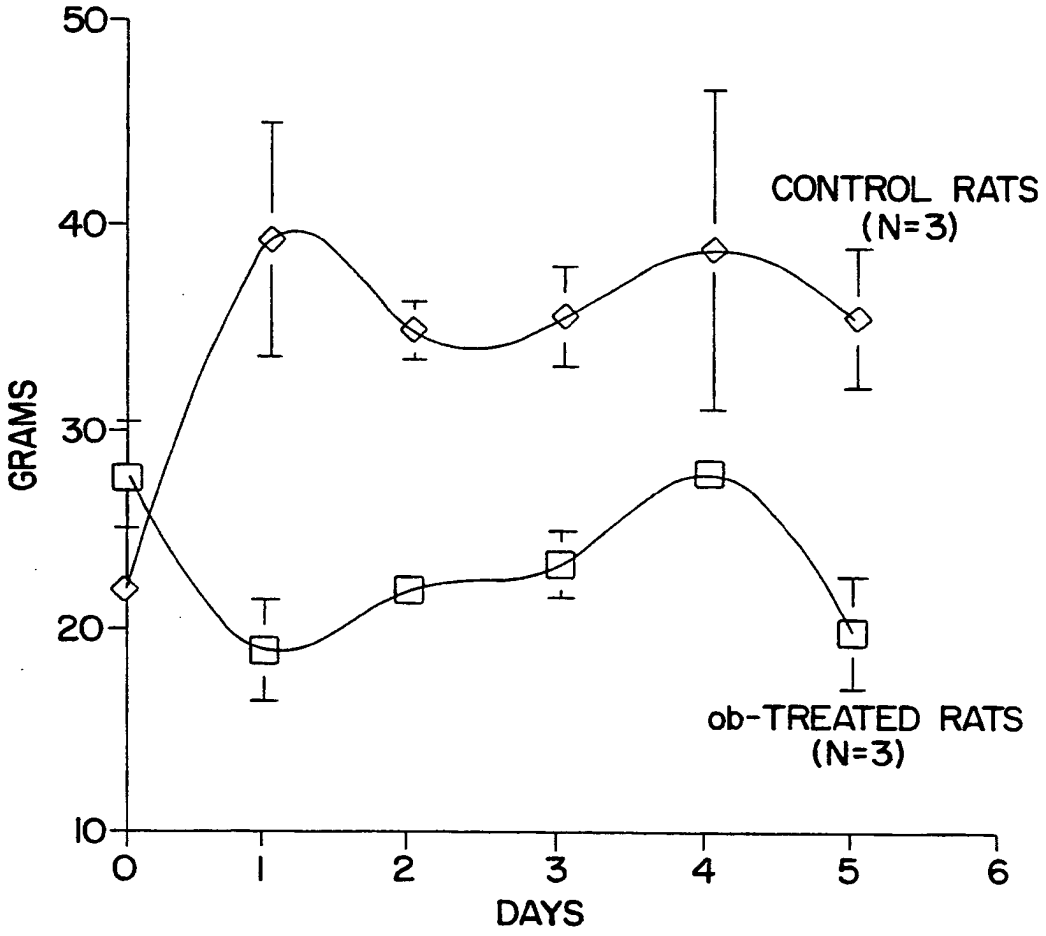


FIG. 4

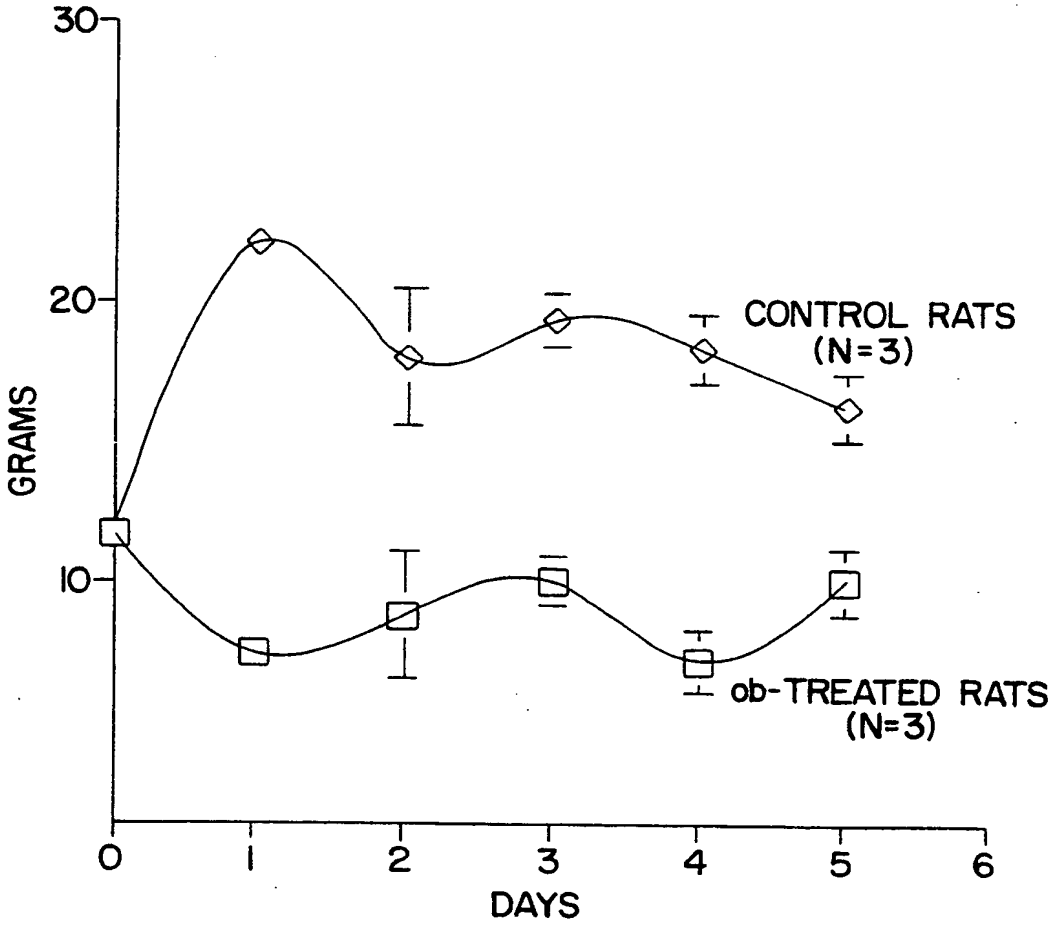


FIG. 5

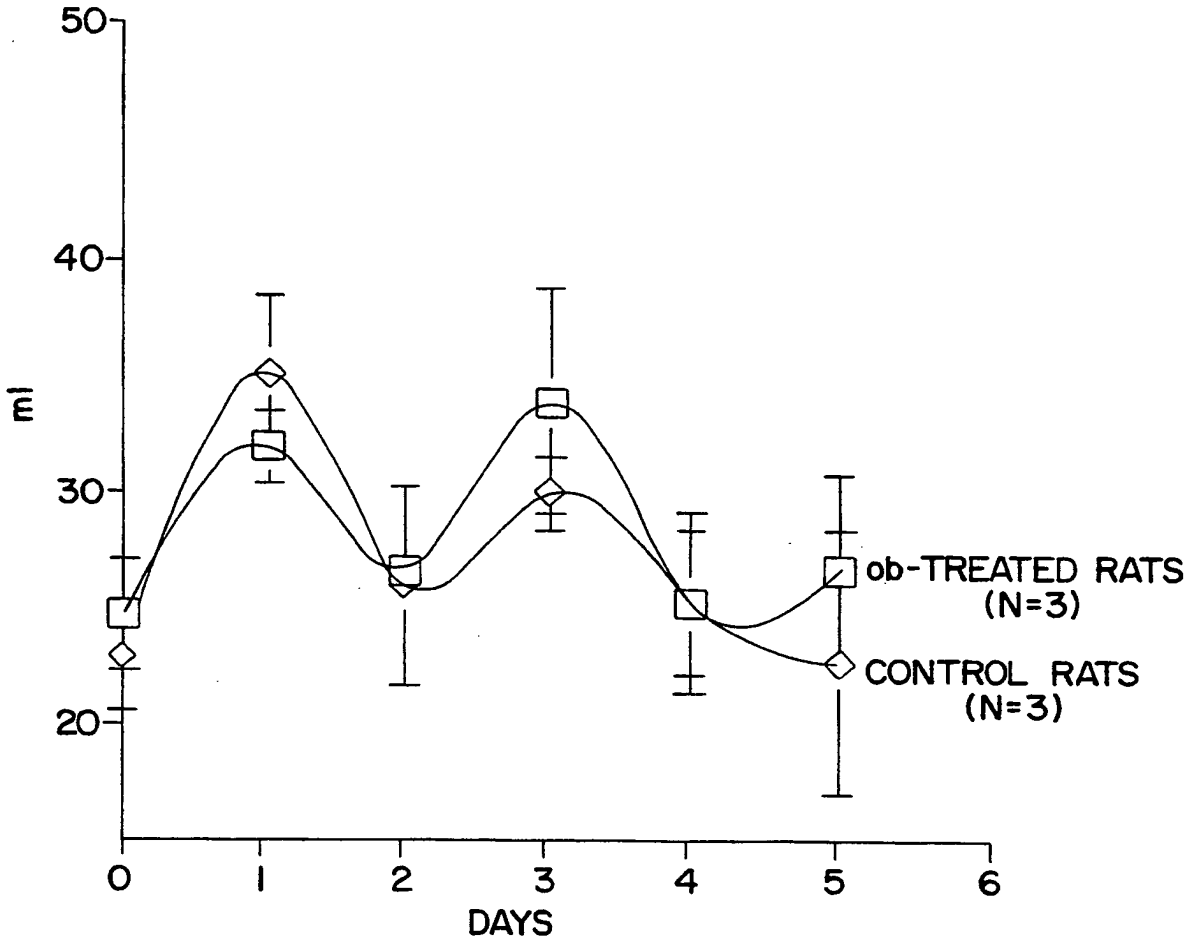


FIG. 6

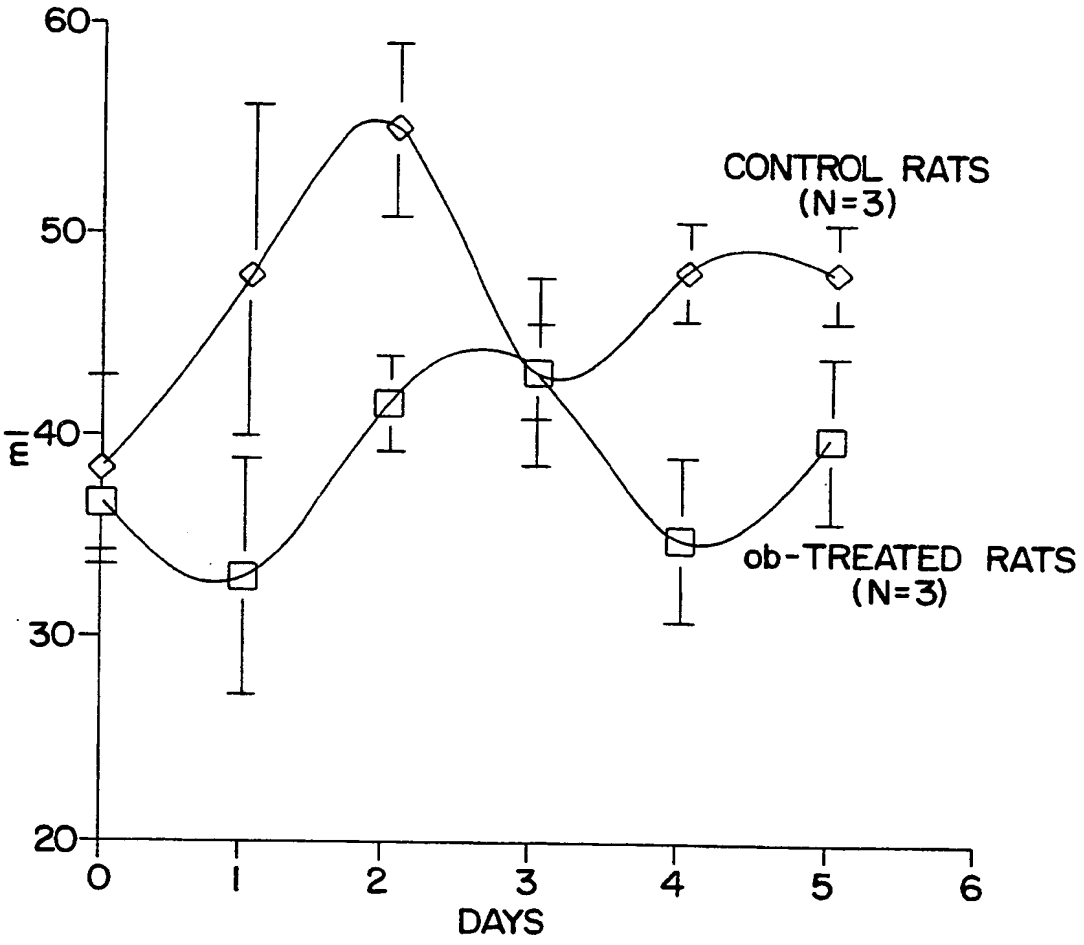
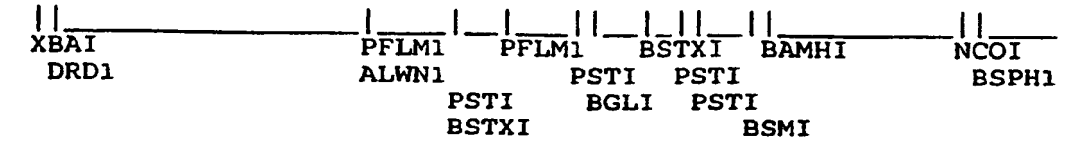


FIG. 7

Argument Map in DNA Strand 1122
from the '/arp/lib/6mers' file.
Translation shown at frame 1.



XbaI

SerArgMetCysTrpArgProLeuCysArgPheLeuTrpLeuTrpSerTyrLeuSerTyr
1 TCTAGAATGTGCTGGAGACCCCTGTGTCGGTTCCTGTGGCTTTGGTCCTATCTGTCTTAT
AGATCTTACACGACCTCTGGGGACACAGCCAAGGACACCGAAACCAGGATAGACAGAATA

1 XBAI, 17 DRD1,

ValGlnAlaValProIleGlnLysValGlnAspAspThrLysThrLeuIleLysThrIle
61 GTTCAAGCAGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATT
CAAGTTCGTCACGGATAGCTCTTTCAGGTCCTACTGTGGTTTTGGGAGTAGTTCTGGTAA

ValThrArgIleAsnAspIleSerHisThrGlnSerValSerAlaLysGlnArgValThr
121 GTCACCAGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGGGTCACT
CAGTGGTCCTAGTTACTGTAAAGTGTGTGCGTCAGCCATAGGCGGTTCGTCTCCCAGTGA

GlyLeuAspPheIleProGlyLeuHisProIleLeuSerLeuSerLysMetAspGlnThr
181 GGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGACCAGACT
CCGAACCTGAAGTAAGGACCCGAAGTGGGGTAAGACTCAAACAGGTTCTACCTGGTCTGA

234 PFLM1, 235 ALWN1,

LeuAlaValTyrGlnGlnValLeuThrSerLeuProSerGlnAsnValLeuGlnIleAla
241 CTGGCAGTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCC
GACCGTCAGATAGTTGTCCAGGAGTGGTCGGACGGAAGGGTTTTACACGACGTCTATCGG

289 PSTI, 299 BSTXI,

AsnAspLeuGluAsnLeuArgAspLeuLeuHisLeuLeuAlaPheSerLysSerCysSer
301 AATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGCTGCTCC
TTACTGGACCTCTTAGAGGCTCTGGAGGAGGTAGACGACCGGAAGAGGTTCTCGACGAGG

330 PFLM1,

LeuProGlnThrSerGlyLeuGlnLysProGluSerLeuAspGlyValLeuGluAlaSer
361 CTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAAGCCTCA
GACGGAGTCTGGTCACCGGACGTCTTCGGTCTCTCGGACCTACCGCAGGACCTTCGGAGT

379 PSTI, 395 BGLI,

LeuTyrSerThrGluValValAlaLeuSerArgLeuGlnGlySerLeuGlnAspIleLeu
421 CTCTACTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTT
GAGATGAGGTGTCTCCACCACCGAAACTCGTCCGACGTCCCGAGAGACGTCCTGTAAGAA

428 BSTXI, 454 PSTI, 466 PSTI,

FIG. 8A

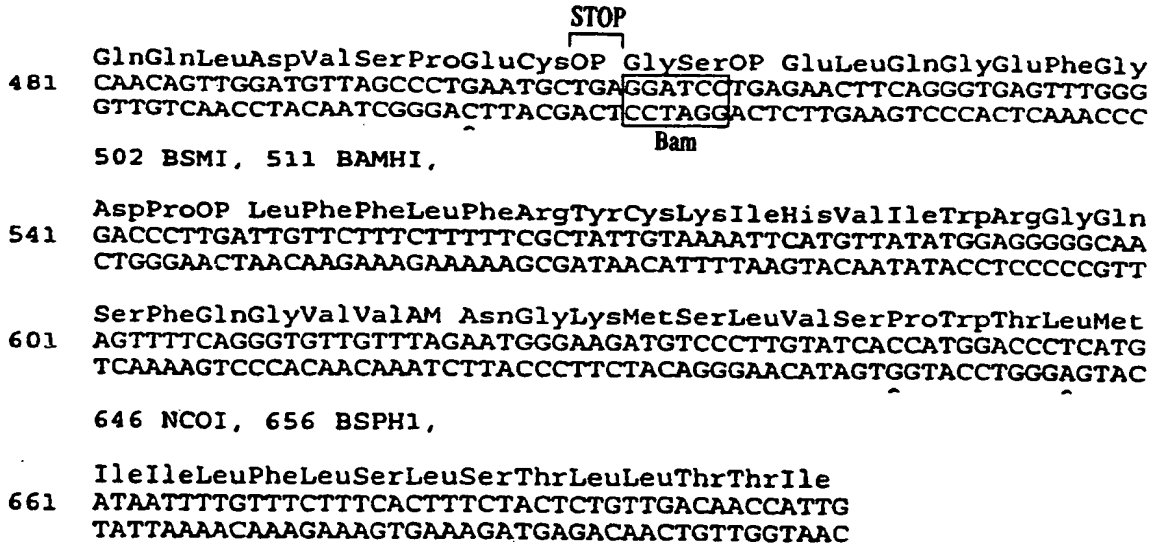

STOP
GlnGlnLeuAspValSerProGluCysOP GlySerOP GluLeuGlnGlyGluPheGly
481 CAACAGTTGGATGTTAGCCCTGAATGCTGAGGATCCTGAGAACTTCAGGGTGAGTTTGGG
GTTGTCAACCTACAATCGGGACTTACGACTCCTAGGACTCTTGAAGTCCCACTCAAACCC
Bam
502 BSMI, 511 BAMHI,
AspProOP LeuPhePheLeuPheArgTyrCysLysIleHisValIleTrpArgGlyGln
541 GACCCTTGATTGTTCTTTCTTTTTCGCTATTGTAAAATTCATGTTATATGGAGGGGGCAA
CTGGGAACTAACAAGAAAGAAAAAGCGATAACATTTTAAGTACAATATACCTCCCCCGTT
SerPheGlnGlyValValAM AsnGlyLysMetSerLeuValSerProTrpThrLeuMet
601 AGTTTTCAGGGTGTTGTTTAGAATGGGAAGATGTCCCTTGTATCACCATGGACCCTCATG
TCAAAAGTCCCACAACAAATCTTACCCTTCTACAGGGAACATAGTGGTACCTGGGAGTAC
646 NCOI, 656 BSPH1,
IleIleLeuPheLeuSerLeuSerThrLeuLeuThrThrIle
661 ATAATTTTGTTTCTTTCACTTTCTACTCTGTTGACAACCATTG
TATTAAAACAAAGAAAGTGAAAGATGAGACAACTGTTGGTAAC

FIG. 8B

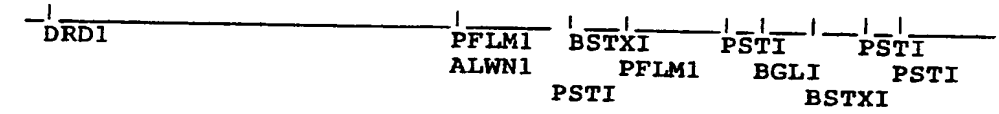
SEQUENCE NAMES, THEIR LENGTHS and % ALIGNMENTS

#1 :	1130	660	98.33%
#2 :	1131	717	90.52%
#3 :	1132	702	92.45%

```
                                                                  XbaI
  0  ..... [CTTCGGCCAGCGCCTTGTAGAAGCGCGTATGGCTTCTAGACATATG] gtgcc
  0  gcact [CTTCGGCCAGCGCCTTGTAGAAGCGCGTATGGCTTCTAGACATATG] .....
  0  gcact [CTTCGGCCAGCGCCTTGTAGAAGCGCGTATGGCTTCTAGACATATG] gcagt

 51  tatc... [CAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACCAGG
 51  ....... [CAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACCAGG
 56  gcctatc [CAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACCAGG

106  ATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGGGTCACTGGCTTGGAC
102  ATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGGGTCACTGGCTTGGAC
114  ATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGGGTCACTGGCTTGGAC

166  TTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCAGTC
162  TTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCAGTC
174  TTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCAGTC

226  TATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCCAATGACCTG
222  TATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCCAATGACCTG
234  TATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCCAATGACCTG

286  GAGAATCTCCGAGACCTCCTCCATCTG] c [TGGCCTTCTCCAAGAGCTGCTCCCTGCC
282  GAGAATCTCCGAGACCTCCTCCATCTG] c [TGGCCTTCTCCAAGAGCTGCTCCCTGCC
294  GAGAATCTCCGAGACCTCCTCCATCTG] g [TGGCCTTCTCCAAGAGCTGCTCCCTGCC

342  TCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAAGCCTCACTCTA
338  TCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAAGCCTCACTCTA
350  TCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAAGCCTCACTCTA
 *

402  CTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACA
398  CTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACA
410  CTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACA

                                  Sma                    myc-tag
462  GTTGGATGTTAGCCCTGAATGCCCCGGGGGTGAGCAGAAGCTGATTTCCGAGGAGGACCT
458  GTTGGATGTTAGCCCTGAATGCCCCGGGGGTGAGCAGAAGCTGATTTCCGAGGAGGACCT
470  GTTGGATGTTAGCCCTGAATGCCCCGGGGGTGAGCAGAAGCTGATTTCCGAGGAGGACCT
       STOP  Bam
522  GAACTGAGGATCCTGAGAACTTCAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTT
518  GAACTGAGGATCCTGAGAACTTCAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTT
530  GAACTGAGGATCCTGAGAACTTCAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTT

582  TCGCTATTGTAAAATTCATGTTATATGGAGGGGG] g [AAAGTTTTCAGGGTGTTGTTT
578  TCGCTATTGTAAAATTCATGTTATATGGAGGGGG] c [AAAGTTTTCAGGGTGTTGTTT
590  TCGCTATTGTAAAATTCATGTTATATGGAGGGGG] c [AAAGTTTTCAGGGTGTTGTTT

638  AGAATGGGAAGATGTCCCTTGT] .....
634  AGAATGGGAAGATGTCCCTTGT] atcaccatggaccctcatgataattgggtttcttac
646  AGAATGGGAAGATGTCCCTTGT] atcaccatggaccctcatgataattgggtttctt..

660  ..........
692  actttctactctgttgacaaccaat
702  ..........
```

FIG. 9

Argument Map in DNA Strand 1119-2.t7
from the '/arp/lib/6mers' file.
Translation shown at frame 1.

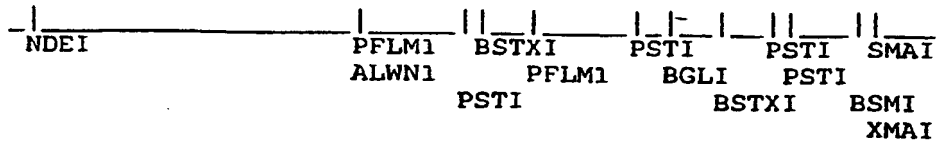



```
    MetCysTrpArgProLeuCysArgPheLeuTrpLeuTrpSerTyrLeuSerTyrValGln
  1 ATGTGCTGGAGACCCCTGTGTCGGTTCCTGTGGCTTTGGTCCTATCTGTCTTATGTTCAA
    TACACGACCTCTGGGGACACAGCCAAGGACACCGAAACCAGGATAGACACAATACAAGTT

    11 DRD1,

    AlaValProIleGlnLysValGlnAspAspThrLysThrLeuIleLysThrIleValThr
 61 GCAGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACC
    CGTCACGGATAGGTCTTTCAGGTCCTACTGTGGTTTTGGGAGTAGTTCTGGTAACAGTGG

    ArgIleAsnAspIleSerHisThrGlnSerValSerAlaLysGlnArgValThrGlyAM
121 AGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGGGTCACTGGCTAG
    TCCTAGTTACTGTAAAGTGTGTGCGTCAGCCATAGGCGGTTCGTCTCCCAGTGACCGATC

    AspPheIleProGlyLeuHisProIleLeuSerLeuSerLysMetAspGlnThrLeuAla
181 GACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCA
    CTGAAGTAAGGACCCGAAGTGGGGTAAGACTCAAACAGGTTCTACCTGGTCTGAGACCGT

    228'PFLM1, 229 ALWN1,

    ValTyrGlnGlnValLeuThrSerLeuProSerGlnAsnValLeuGlnIleAlaAsnAsp
241 GTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCCAATGAC
    CAGATAGTTGTCCAGGAGTGGTCGGACGGAAGGGTTTTACACGACGTCTATCGGTTACTG

    283 PSTI, 293 BSTXI,

    LeuGluAsnLeuArgAspLeuLeuHisLeuLeuAlaPheSerLysSerCysSerLeuPro
301 CTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGCTGCTCCCTGCCT
    GACCTCTTAGAGGCTCTGGAGGAGGTAGACGACCGGAAGAGGTTCTCGACGAGGGACGGA

    324 PFLM1,

    GlnThrSerGlyLeuGlnLysProGluSerLeuAspGlyValLeuGluAlaSerLeuTyr
361 CAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAAGCCTCACTCTAC
    GTCTGGTCACCGGACGTCTTCGGTCTCTCGGACCTACCGCAGGACCTTCGGAGTGAGATG

    373 PSTI, 389 BGLI,

    SerThrGluValValAlaLeuSerArgLeuGlnGlySerLeuGlnAspIleLeuGlnGln
  1 TCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGACATTCTTCAACAG
    AGGTGTCTCCACCACCGAAACTCGTCCGACGTCCCGAGAGACGTCCTGTAAGAAGTTGTC

    422 BSTXI, 448 PSTI, 460 PSTI,
```

PREMATURE STOP COLON

FIG. 10

Argument Map in DNA Strand 1150
from the '/arp/lib/6mers' file.
Translation shown at frame 3.



Nde

GlyAspIleHisMetValProIleGlnLysValGlnAspAspThrLysThrLeuIleLys
3 GGAGATATACATATGGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAG
CCTCTATATGTATACCACGGATAGGTCTTTCAGGTCCTACTGTGGTTTTGGGAGTAGTTC

12 NDEI,

ThrIleValThrArgIleAsnAspIleSerHisThrGlnSerValSerAlaLysGlnArg
63 ACCATTGTCACCAGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGG
TGGTAACAGTGGTCCTAGTTACTGTAAAGTGTGTGCGTCAGCCATAGGCGGTTCGTCTCC

ValThrGlyLeuAspPheIleProGlyLeuHisProIleLeuSerLeuSerLysMetAsp
123 GTCACTGGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGAC
CAGTGACCGAACCTGAAGTAAGGACCCGAAGTGGGGTAAGACTCAAACAGGTTCTACCTG

182 PFLM1,

GlnThrLeuAlaValTyrGlnGlnValLeuThrSerLeuProSerGlnAsnValLeuGln
183 CAGACTCTGGCAGTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAG
GTCTGAGACCGTCAGATAGTTGTCCAGGAGTGGTCGGACGGAAGGGTTTTACACGACGTC

183 ALWN1, 237 PSTI,

IleAlaAsnAspLeuGluAsnLeuArgAspLeuLeuHisLeuLeuAlaPheSerLysSer
243 ATAGCCAATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGC
TATCGGTTACTGGACCTCTTAGAGGCTCTGGAGGAGGTAGACGACCGGAAGAGGTTCTCG

247 BSTXI, 278 PFLM1,

CysSerLeuProGlnThrSerGlyLeuGlnLysProGluSerLeuAspGlyValLeuGlu
303 TGCTCCCTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAA
ACGAGGGACGGAGTCTGGTCACCGGACGTCTTCGGTCTCTCGGACCTACCGCAGGACCTT

327 PSTI, 343 BGLI,

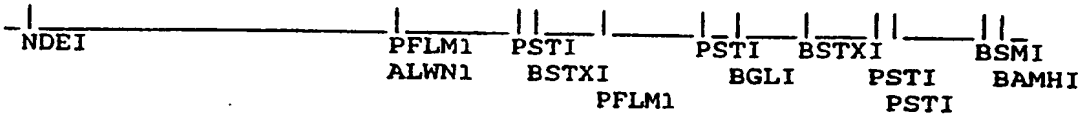
AlaSerLeuTyrSerThrGluValValAlaLeuSerArgLeuGlnGlySerLeuGlnAsp
363 GCCTCACTCTACTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGAC
CGGAGTGAGATGAGGTGTCTCCACCACCGAAACTCGTCCGACGTCCCGAGAGACGTCCTG

376 BSTXI, 402 PSTI, 414 PSTI,

IleLeuGlnGlnLeuAspValSerProGluCysProGlyGlyArgArgAlaSerGly
423 ATTCTTCAACAGTTGGATGTTAGCCCTGAATGCCCCGGGGGACGGAGAGCTTCCGGGG
TAAGAAGTTGTCAACCTACAATCGGGACTTACGGGGCCCCCTGCCTCTCGAAGGCCCC CONTINUES W/
SmaI Myctag
-NOT INCLUDED-

FIG. 12

Argument Map in DNA Strand 1127-
from the '/arp/lib/6mers' file.
Translation shown at frame 3.



Nde

GlyAspIleHisMetValProIleGlnLysValGlnAspAspThrLysThrLeuIleLys
3 GGAGATATACATATGGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAG
CCTCTATATGTATACCACGGATAGGTCTTTCAGGTCCTACTGTGGTTTTGGGAGTAGTTC

12 NDEI,

ThrIleValThrArgIleAsnAspIleSerHisThrGlnSerValSerAlaLysGlnArg
63 ACCATTGTCACCAGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAGG
TGGTAACAGTGGTCCTAGTTACTGTAAAGTGTGTGCGTCAGCCATAGGCGGTTCGTCTCC

ValThrGlyLeuAspPheIleProGlyLeuHisProIleLeuSerLeuSerLysMetAsp
123 GTCACTGGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGAC
CAGTGACCGAACCTGAAGTAAGGACCCGAAGTGGGGTAAGACTCAAACAGGTTCTACCTG

182 PFLM1,

GlnThrLeuAlaValTyrGlnGlnValLeuThrSerLeuProSerGlnAsnValLeuGln
183 CAGACTCTGGCAGTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAG
GTCTGAGACCGTCAGATAGTTGTCCAGGAGTGGTCGGACGGAAGGGTTTTACACGACGTC

183 ALWN1, 237 PSTI,

IleAlaAsnAspLeuGluAsnLeuArgAspLeuLeuHisLeuLeuAlaPheSerLysSer
243 ATAGCCAATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGC
TATCGGTTACTGGACCTCTTAGAGGCTCTGGAGGAGGTAGACGACCGGAAGAGGTTCTCG

247 BSTXI, 278 PFLM1,

CysSerLeuProGlnThrSerGlyLeuGlnLysProGluSerLeuAspGlyValLeuGlu
303 TGCTCCCTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGAA
ACGAGGGACGGAGTCTGGTCACCGGACGTCTTCGGTCTCTCGGACCTACCGCAGGACCTT

327 PSTI, 343 BGLI,

AlaSerLeuTyrSerThrGluValValAlaLeuSerArgLeuGlnGlySerLeuGlnAsp
363 GCCTCACTCTACTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGAC
CGGAGTGAGATGAGGTGTCTCCACCACCGAAACTCGTCCGACGTCCCGAGAGACGTCCTG

376 BSTXI, 402 PSTI, 414 PSTI,

STOP

IleLeuGlnGlnLeuAspValSerProGluCysOP GlySer
423 ATTCTTCAACAGTTGGATGTTAGCCCTGAATGCTGAGGATCC
TAAGAAGTTGTCAACCTACAATCGGGACTTACGACTCCTAGG

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter. ıal Application No
PCT/US 96/06609

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/85 C12N5/10 C12P21/02 A61K48/00
C07K14/575 C07K14/72 C07K16/26 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P A61K C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, vol. 272, 1994, pages 425-432, XP002008560 Y. ZHANG ET AL.: "Positional cloning of the mouse obese gene and its human homologue" cited in the application see the whole document. ---	1-7,9-38
X	BIOCHEM. BIOPHYS. RES. COMMUN., vol. 209, 26 April 1995, pages 944-952, XP002008561 T. MURAKAMI ET AL.: "Cloning of rat obese cDNA and its expression in obese rats" see the whole document. --- -/--	1-7,9-38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 1996	06.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Yeats, S

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/06609

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DIABETOLOGIA, vol. 14, 1978, pages 141-148, XP002008562 D.L. COLEMAN ET AL.: "Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice" cited in the application see the whole document. ---	1
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P,X	WO,A,96 05309 (THE ROCKEFELLER UNIVERSITY) 22 February 1996 see page 15, lines 20-26, page 31, lines 7-13, pages 90-93 and Claims. -----	1-7,9-38

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
PCT/US 96/06609

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE-A- 19531931	07-03-96
		GB-A- 2292382	21-02-96